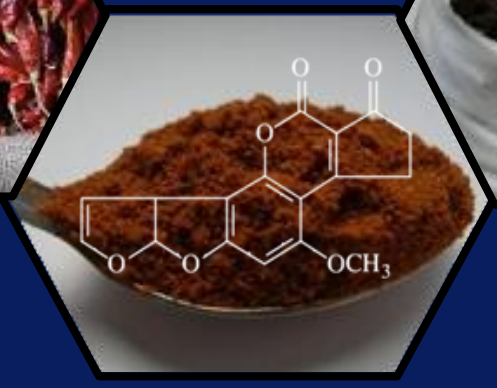
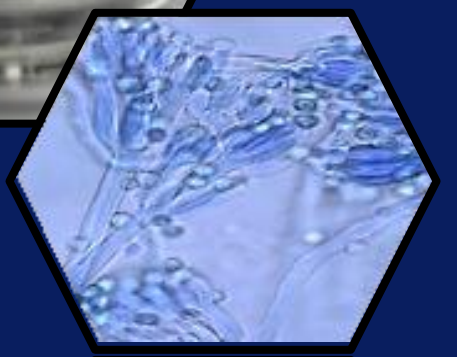
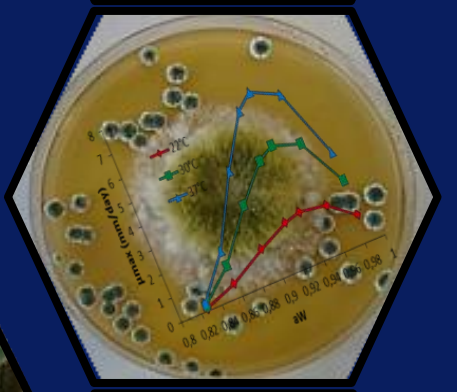
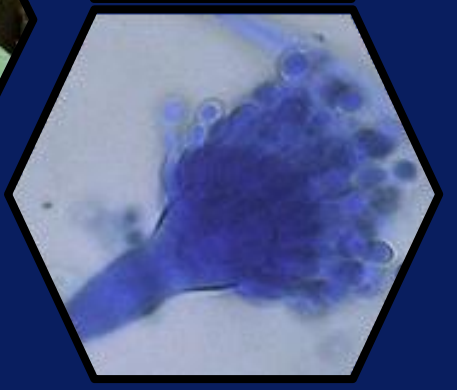




RISK ASSESSMENT OF MYCOTOXINS AND PREDICTIVE MYCOLOGY IN SRI LANKAN SPICES: CHILLI AND PEPPER

Pratheeba Yogendrarajah (MSc.)



UNIVERSITEIT GENT

FACULTY OF BIOSCIENCE ENGINEERING

“Every struggle in your life has shaped you into the person you are today.
Be thankful for the hard times, they can only make you stronger”

RISK ASSESSMENT OF MYCOTOXINS AND PREDICTIVE MYCOLOGY IN SRI LANKAN SPICES: CHILLI AND PEPPER

Thesis submitted in fulfillment of the requirements for the degree of Doctor (PhD) in Applied Biological Sciences: Chemistry and Bioprocess Technology

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Laboratory of Applied Mycology, Department of Applied Biosciences, Faculty of Bioscience Engineering, Ghent University

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Pratheeba

13th of March, 2015, Ghent

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NOMENCLATURE OF ABBREVIATIONS AND ACRONYMS

3-ADON	3-acetyldeoxynivalenol
15-ADON	15-acetyldeoxynivalenol
Af	Accuracy factor
AfAp	*Aspergillus flavus* and/or *Aspergillus parasiticus*
AFB1	Aflatoxin B1
AFB2	Aflatoxin B2
AFG1	Aflatoxin G1
AFG2	Aflatoxin G2
AFPA	Aspergillus flavus parasiticus agar
AFs	Aflatoxins
ALARA	As low as reasonably achievable
AME	Alternariol methyl ether
ANOVA	Analysis of variance
AOAC	Association of Official Analytical Chemists
AOH	Alternariol
AR	Absolute response
ASTA	American Spice Trade Association
a_w	Water activity
B_f	Bias factor
BMD	Bench mark dose
BMDL	BMD lower confidence limit
$BMDL_{10}$	BMD for a 10% increase in cancer incidence
BEN	Balkan endemic nephropathy
BET	Brunauer-Emmett-Teller
BW/bw	Body weight
CAST	Council for Agriculture Science and Technology
CFU	Colony forming unit
CIA	Central Intelligence Agency
CIT	Citrinin
CO	Cut-off
CONTAM	Scientific Panel on Contaminants in the Food chain of EFSA
χ^2	Reduced chi-square
DG	Gibbs free energy change (J/mol)
ΔH	Enthalpy (kJ/mol)
ΔH_B	Binding energy (J/mol)
ΔH_{vap}	Heat of vaporisation (kJ/mol/K)
DEA	Department of Export Agriculture
DLP	Double log polynomial
DON	Deoxynivalenol
DOA	Department of Agriculture
d-SPE	Dispersive solid phase extraction
DS	Sorption entropy (J/mol/K)
dwb	Dry weight basis
R_{ave}	Average of the residuals
EC	European Commission
EFSA	European Food Safety Authority
EMC	Equilibrium moisture content
$EMC_{exp,i}$	i^{th} value of the experimentally measured EMC
$EMC_{pred,i}$	i^{th} value of the predicted EMC
Eq.	Equation

ESA	European Spice Association
ESI	Electrospray ionization
EU	European Union
FAO	Food and Agriculture Organization
FASFC	Federal Agency for the Safety of the Food Chain
FB1	Fumonisin B1
FB2	Fumonisin B2
FB3	Fumonisin B3
FD	Fluorescence Detector
FFQ	Food frequency questionnaire
GAB	Guggenheim-Anderson-de Boer
GCB	Graphitized carbon black
GAP	Good agriculture practices
GMP	Good manufacturing practices
HACCP	Hazard analysis critical control point
HBGV	Health based guidance value
HBsAg (+)ive	Hepatitis B surface antigen positive
HBsAg (-)ive	Hepatitis B surface antigen negative
HBV	Hepatitis B virus
HCC	Hepatocellular carcinoma
HPLC	High performance liquid chromatography
HRMS	High resolution mass spectrometry
IARC	International Agency for Research on Cancer
IPC	International Pepper Community
IQR	Interquartile range
IS	Internal standard
JECFA	Joint FAO/WHO Expert Committee on Food Additives
LB	Lower bound
LC-MS/MS	Liquid chromatography tandem mass-spectrometry
LDC	Less developed countries
LOD	Limit of detection
LOQ	Limit of quantification
MB	Medium bound
MC	Moisture content
MEA	Malt extract agar
ME	Matrix effect
MeCN	Acetonitrile
MeOH	Methanol
ML	Maximum level
M_0	Monolayer moisture content (% dwb)
MoE	Margin of exposure
MMC	Matrix matched calibration curve
MRM	Multiple reaction monitoring
MT	Metric ton
MW	Molecular weight
m/z	Mass to charge ratio
μ_{max}	Maximal radial growth rate (mm/day)
N	Number of observations or data points
n	Number of samples or constants
NA	Not applicable
ND	Not-detected/non-detects

NG	No growth
NEO	Neosolaniol
NOAEL	No observed adverse effect level
OMST	O-methyl sterigmatocystin
OTA	Ochratoxin A
P (%)	Mean relative percentage deviation modulus
PBS	Phosphate-buffered saline
PMTDI	Provisional maximum tolerable daily intake
PTWI	Provisional tolerable weekly intake
PoD	Point of departure
PSA	Primary secondary amine
q_{st}	Net isosteric heat of sorption or enthalpy of sorption (kJ/mol)
Q_{st}	Total heat of sorption (kJ/mol)
QuEChERS	Quick, Easy, Cheap, Effective, Rugged and Safe
R	Universal gas constant (8.314 J/mol/K)
RASFF	Rapid Alert System for Food and Feed
R^2	Coefficient of determination
RH	Relative humidity
RMSE	Root mean square error
RR	Relative response
RSD	Relative standard deviation
RSDr	Intra-day repeatability
RSDR	Inter-day reproducibility/intermediate precision
RSS	Residual sums of squares
S	Solid surface Area (m^2/g solids)
SCF	Scientific Committee on Food
SD	Standard deviation
SEP	Standard error of prediction
S/N	Signal to noise ratio
SPE	Solid phase extraction
SQRT	Square root
SSE	Signal suppression-enhancement effect
SLS	Sri Lankan Standards
STERIG	Sterigmatocystin
T	Temperature (Kelvin or degree Celsius)
TCA	Tri-carboxylic acid
TDI	Tolerable daily intake
tR	Retention time
UB	Upper bound
USDA	United States Department of Agriculture
WHO	World Health Organization
wwb	Wet weight basis
ZAN	Zearalanone
ZEN	Zearalenone

RESEARCH FRAMEWORK AND OBJECTIVES

The food industry is currently one of the fastest growing in the world, as populations are burgeoning worldwide and global trading of food is expensing. Since agriculture is the main industry of economics in most developing nations, food is what these countries have to offer as their best trade asset. However, food safety is one of the most ignored areas of policy and less frequently voiced topic in these low-income countries. In these countries, focus has long been made on food security. Food systems in these countries are not always well organized and comprehensive as in the industrialized world. This situation is being intensified by several pressures such as an ever-growing population, rapid urbanization, climate change and most importantly lack of economic and technical resources needed to support an organized food safety system. As a result, people in developing countries are continuously exposed to a wider range of potential food safety risks than those living in wealthier countries. Foodborne illnesses rise at unacceptable rates, while new hazards continue to enter the food supply.

In this regard, mycotoxins still remain as an evolving food safety challenge to the farm to fork continuum. Mycotoxins are toxic secondary fungal metabolites produced by fungi, causing mycotoxicosis. They are considered as unavoidable natural contaminants in agricultural commodities, produced in the field or during post-harvest. The toxic effects of mycotoxins are huge and being researched comprehensively in different countries. Despite the wide range of studies on the occurrence of mycotoxins in several food products in various countries, only limited data are available on mycotoxin exposure among different populations. There are significant differences in mycotoxins occurrence between countries and even within countries with regard to the food intake, thus leading risk assessment to be country specific. Moreover, compared to Africa and Europe, mycotoxin occurrence and subsequent quantitative exposure assessment studies are rather limited in the Asian context. The issue of mycotoxins in Sri Lanka is not known.

Located strategically in the Indian ocean, Sri Lanka “the spice island’’, was a hub in the maritime silk and spice routes for millennia. It drew traders from the east and west for both business and pleasure. Notable among the attractions were spices, whose many aromas and flavors formed an integral part of the tropical paradise experience. The spice sector plays an important role in the Sri Lankan economy. However, there are a number of interrelated issues and challenges in relation to the cultivation practices, technology and marketing in this sector. The inferior quality of spice and allied products has been a serious issue in the present context of high quality consciousness. The prevailing climatic conditions, high moisture retention, contamination by microorganisms such as moulds and the interlinked presence of mycotoxins could be common problems affecting the quality of spices in Sri Lanka. Beyond poor weather conditions, they are mainly attributed to low cost processing methods (open air sun drying), poor storage facilities, small scale nature of production

units and early harvesting habits to meet family cash needs of resource-poor farmers. Moreover, it is conceivable that the Lankan spices need to undergo heavy re-processing to improve their quality before export, particularly to comply with trade regulations enforced by the major importing countries, the EU and USA.

Among the spices, chilli and pepper have been reported to be frequently contaminated with mycotoxins, aflatoxins and ochratoxins. Moreover, the level of fungal contamination in pepper has been described to be the highest for spices. Spices are ubiquitously used in Sri Lankan cooking and Lankan preparations are believed to be among the world's hottest in terms of chilli content. All the above mentioned facts, stimulated the interest to study the mycotoxin problems in these spices produced and consumed in Sri Lanka, given the lack of information on the mycotoxin problem in the island.

Consequently, a research framework to study the mycotoxins issues in two major Sri Lankan spices was developed, more specifically in pepper (*Piper nigrum* L.) and pepper products and in chilli (*Capsicum annum* L.) and allied products.

The major objectives of this PhD thesis are the following:

1) To optimize a multi-mycotoxin analytical method for the selected spice matrices using liquid chromatography tandem mass spectrometry (LC-MS/MS) .....[Chapter 2]

2) To apply this method on samples collected locally throughout the spices production chain (farm-to-fork).....[Chapters 3 and 4]

3) To perform a quantitative risk assessment on mycotoxins due to the consumption of selected spices in Sri Lanka.....[Chapter 5]

4) To study the relationship between mould growth and mycotoxin production on selected spices in order to develop predictive models relating mould growth and mycotoxin production as function of intrinsic and extrinsic factors.....[Chapters 6, 7 & 8]

5) To identify a possible mycotoxin mitigation strategy to tackle the mycotoxin problem .....[Chapter 9]

The schematic outline of this PhD research is given in Fig. I.

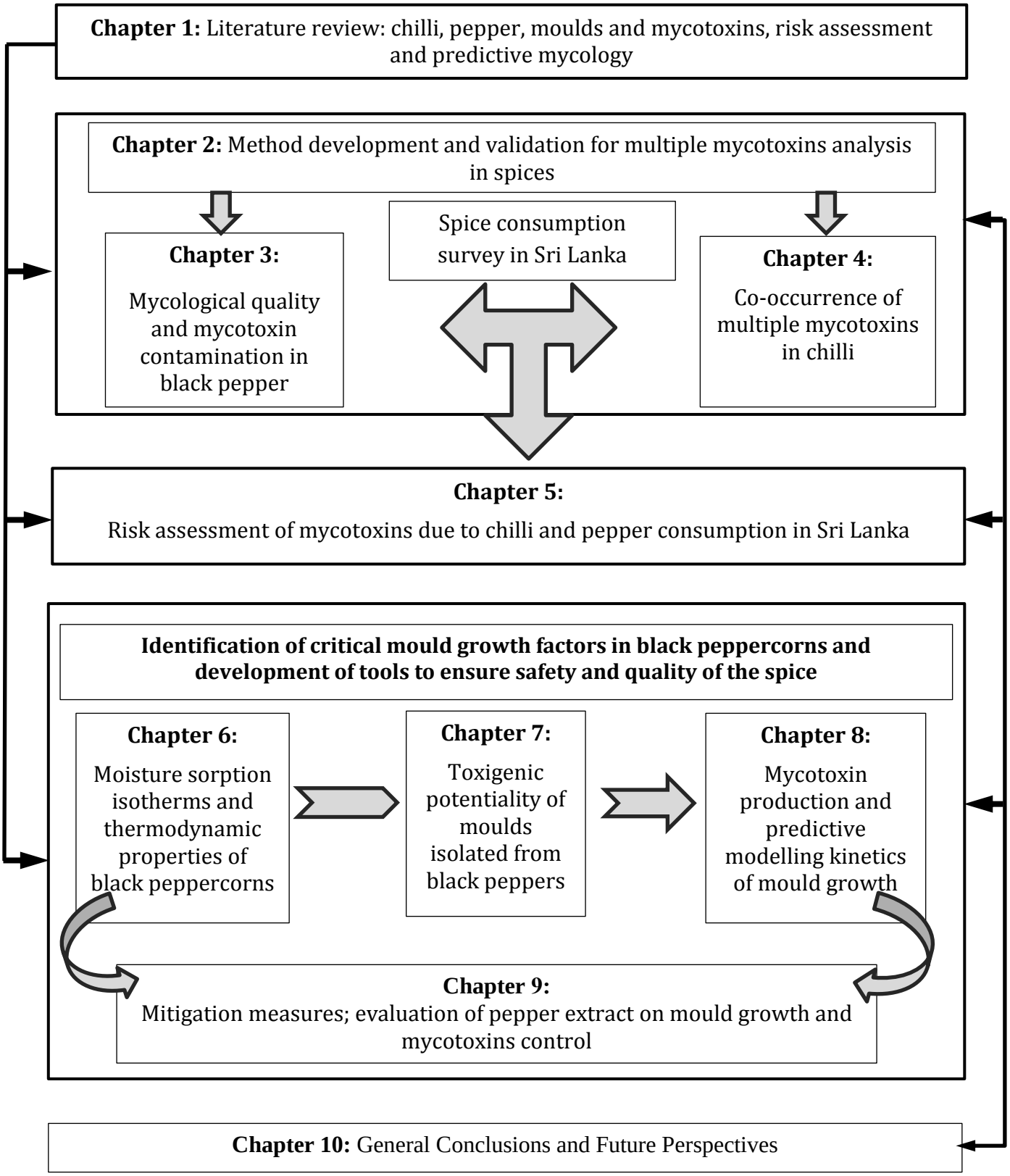


Fig. I. Schematic outline of this PhD research.

SUMMARY

Sri Lanka, "The Pearl of Indian Ocean" in South Asia is also known as "Spice Island", where highly priced spices are produced, consumed and exported. Spices are well known for imparting flavour, colour, aroma in diverse cuisines and for therapeutic properties. However, their quality is often compromised. As a developing country Sri Lanka has its own limitations in producing high quality spices for local consumption and to comply with trade regulations enforced by the importing countries. Moreover, as a tropical nation the prevailing climatic conditions, while supporting the spice crop development could also be highly favorable for mould infestation and mycotoxin contamination in the field or during post-harvest practices. Mycotoxins are toxic secondary metabolites produced by diverse filamentous fungi. Like many other foods, spices could also be contaminated with moulds and mycotoxins affecting their safety and quality. Hence, this research work is carried out in order to identify the actual situation in moulds and mycotoxin contamination in spices and to perform a quantitative risk assessment of mycotoxins in Sri Lanka, given the limited information on the mycotoxin issues in this island. In this study, chilli (*Capsicum annum* L.) and pepper (*Piper nigrum* L.) were selected since these two are the most important spices of world trade and consumption.

Chapter 1 reviews concisely the relevant literature. The findings of this PhD research are described in eight chapters, which contributed to eight scientific publications **(Chapter 2-9).**

In **Chapter 1** some contextual information on spices, chilli and pepper, uses, production and trade statistics are given. A concise description on chemistry, associated mould species, occurrence and toxicity of some important mycotoxins is overviewed. Moreover, their analytical detection techniques are briefed. The principles of risk assessment with special focus on mycotoxins are discussed. Finally, the study on predictive modelling of fungal growth "predictive mycology" is introduced; concepts and applicability of different models are explained.

Chapter 2 describes a reliable and rapid method developed based on a QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe) extraction procedure for the determination of multiple mycotoxins in spices, chilli, black and white peppers. High-performance liquid chromatography tandem mass spectrometry (HPLC-MS/MS) was used for the quantification and confirmation of 17 chemically diverse mycotoxins. Mycotoxins were extracted from the hydrated spices using acidified acetonitrile, followed by partitioning with NaCl and anhydrous $MgSO_4$ excluding the use of dispersive-solid phase extraction. Electrospray ionization at positive mode was applied to simultaneously detect all the mycotoxins in a single run time of 20 min. Multiple reaction monitoring mode, choosing at least two abundant fragment ions per analyte was applied. Recoveries (75 to 117%) were in accordance with the performance criteria required by the European

Commission (EC, 401/2006). The limit of quantification (LOQ) ranged from 2.3 to 146 µg/kg. The method LOQ meets the maximum levels (MLs) of the two regulated mycotoxins, aflatoxins and ochratoxin A (OTA) in spices hence, it could be used for the purpose of enforcement of the EU MLs. The validated method was finally applied to screen mycotoxins in chilli and pepper samples collected from Sri Lanka as described in Chapters 3 and 4.

Chapter 3 reveals the characterization of toxigenic moulds and occurrence of multiple mycotoxins in Sri Lankan peppers. Characterization of the moulds was carried out in *Aspergillus flavus* and *parasiticus* agar (AFPA) and malt extract agar (MEA) in 77 black pepper and 11 white pepper samples. *Aspergillus flavus, Aspergillus parasiticus, Aspergillus niger* and *Penicillium* spp. were found to be the most dominant fungi. In total, 73% of the black pepper and 64% of the white pepper samples were contaminated with *Aspergillus flavus* and/or *Aspergillus parasiticus* (AfAp). The frequency of occurrence of *A. niger* in black pepper was 62% with counts up to $1.3*10^3$ CFU/g. *Penicillium* spp. were found in 61% and 55% of the black and white pepper samples, respectively. Other *Aspergillus* spp., found in peppers included, *Aspergillus terrus, Aspergillus tamarii, Aspergillus candidus, Aspergillus penicilloides, Aspergillus sydowii* and *Aspergillus fumigatus*. Mould counts in black pepper (10^2-10^4 CFU/g) were significantly higher than that of white pepper ($<10^2$ CFU/g). Mould contamination was compared between production steps, forms, sampling regions and grades of pepper.

Apart from the occurrence of "classical mycotoxins" of spices, aflatoxins (<LOQ-17 µg/kg) and OTA (<LOQ-79 µg/kg), other toxins including fumonisin B1 (FB1; <LOQ-135 µg/kg), sterigmatocystin (STERIG; <LOQ-49 µg/kg) and citrinin (CIT; <LOQ-112 µg/kg) were detected in black peppers (n=82). STERIG was detected very frequently (44%). In total, 63% of the aflatoxin B1 (AFB1) positives exceeded the EU ML of 5 µg/kg. Moreover, 63% of the black pepper samples were contaminated with at least one mycotoxin and 12% had more than two toxins. Mycotoxin contamination in white pepper (n=11) was significantly less compared to black pepper. For comparison reasons, some pepper samples from Belgium (n=27) were also analyzed.

Chapter 4 describes the co-occurrence of multiple mycotoxins in dry chilli samples (n=86) collected from Sri Lankan markets. In addition to aflatoxins (<LOQ-718 µg/kg) and OTA (<LOQ-282 µg/kg), the chilli samples were also found to be contaminated with STERIG (<LOQ-32 µg/kg), fumonisin B2 (FB2; <LOQ-87 µg/kg), CIT (<LOQ-2.1 mg/kg) and alternariol methyl ether (70 and 222 µg/kg). AFB1 was the predominant mycotoxin contaminating almost 77% of the samples. Remarkably, 67% of the samples exceeded EU ML of 5 µg/kg for AFB1 and 44% exceeded the EU ML of 10 µg/kg for total aflatoxins. While OTA was found in 41% of the samples notably, 38% of the total samples were contaminated with STERIG. Overall, 87% of the samples, was contaminated

at least with one mycotoxin. One third of the chilli samples were contaminated with more than three different mycotoxins. Co-occurrence of different mycotoxins, AFB1-OTA (36%), AFB1-STERIG (28%), OTA-AFB1-STERIG (17%) and AFB1-FB2 (14%) was found in different forms of chillies. Higher frequency of mycotoxins co-occurrence found in the processed chillies such as flakes and powder could be due to the fraudulent usage of low quality grade chilli pods for processing. For comparison reasons some chilli samples collected from markets in Belgium (n=35) were also analyzed.

Chapter 5 describes the quantitative risk assessment (deterministic and probabilistic) of mycotoxins due to the consumption of chilli and black pepper in Sri Lanka. A food frequency questionnaire was administered in order to collect the data on consumption of spices by households in the Northern and Southern region (n=249). Mean exposure to AFB1 in the North (3.49 ng/kg BW/day) and South (2.13 ng/kg BW/day) have exceeded the proposed tolerable daily intake (1 ng/kg BW/day) due to chilli consumption at the lower bound deterministic scenario, while exposure to OTA was small. Dietary exposure to other mycotoxins, FB1, FB2, STERIG and CIT due to spices were estimated. Margin of exposure estimations at the mean exposure to AFB1 were remarkably lower due to chilli (45-78) than for pepper (2315-10,857). Moreover, the hepato cellular carcinoma (HCC) risk associated with the mean AFB1 exposure through chilli at the lower bound was 0.046 and 0.028 HCC cases/year/100,000 based on the North and South consumption, respectively. AFB1 exposure *via* chilli should be considered as a high public health concern in Sri Lanka due to both high mycotoxin concentration and high consumption.

Chapter 6 explains the moisture sorption isotherms developed for whole black peppercorns at 22, 30 and 37°C using the standard static gravimetric method. The sorption isotherms exhibited a type III behavior according to Brunauer-Emmett-Teller (BET) classifications. The equilibrium moisture content (EMC) decreased with increasing temperature at a particular a_w. Hysteresis existed over the entire a_w range at 30 and 37°C. But at 22°C, an intersection of the curves was found at a_w closer to 0.75. Eleven commonly used sorption isotherm equations were fitted to the experimental sorption data. The Guggenheim-Anderson-de Boer (GAB) and Peleg models were determined to best describe the experimental moisture sorption data. The Oswin, modified Oswin, modified Mizrahi, double log polynomial models were found to adequately describe the experimental data. The monolayer moisture content (M_0), calculated using the GAB models were 3.49-4.78% for adsorption and 4.36-4.67% for desorption. The maximum isosteric heat of sorption was 28.1 and 73.3 kJ/mol for adsorption and desorption, respectively which was found at 4% EMC. The Gibbs free energy change for adsorption ($-\Delta G$) ranged from 100.6-9370.6 J/mol depending on the temperature and EMC (1-40%). The developed sorption isotherms could definitely be used during the industrial drying, processing and storage of whole black peppercorns. The black peppercorns

can be stored at 22, 30 and 37°C, by reducing their moisture content to 10, 8 and 7%, respectively, by simultaneously reducing the a_w below 0.6.

Chapter 7 describes the toxigenicity of *A. parasiticus* and *A. flavus* isolates of Sri Lankan black peppers. An LC-MS/MS method was developed to quantify twenty secondary metabolites that could be produced by different fungal species (*Aspergillus, Penicillium, Fusarium* and *Alternaria* spp.) in malt extract agar. The mycotoxigenic potential of *Aspergillus flavus* (n=11) and *A. parasiticus* (n=6) strains isolated from black peppers following their growth in malt extract agar at 22, 30 and 37°C was assessed using the method developed. All the *A. flavus* isolates produced AFB1 and O-methyl sterigmatocystin (OMST) while 91% produced aflatoxin B2 (AFB2) and 82% of them produced STERIG at 30°C. Except one, all *A. parasiticus* isolates produced all the four aflatoxins, STERIG and OMST at 30°C, but production of aflatoxin G was found to be much lower in this substrate. Following trend was found with mycotoxin production 30°C>22°C>37°C, while growth rate showed a different trend, 30°C>37°C>22°C for both fungal species. Moreover, notable correlations were found between different secondary metabolites of both species.

Chapter 8 discusses the growth and mycotoxin production of an *A. parasiticus* and three *A flavus* isolates in whole black peppercorns using a full factorial design with seven water activity (a_w) (0.826-0.984) levels and three temperatures (22, 30 and 37°C) to develop predictive models. Growth rates and lag phases were estimated using linear regression. Several secondary kinetic models were assessed for their ability to describe the radial growth rate as a function of individual and combined effect of a_w and temperature. Evaluating the statistical indices, the Rosso square root cardinal model can be recommended to best describe the individual a_w effect while extended Gibson model could be best for describing the combined a_w-temperature effect on the growth of both *A. flavus* and *A. parasiticus* species in peppercorns. The bias factors (0.70-1.01), accuracy factors (1.01-1.41) and root mean square error (0.019-0.280) show that the models are conservative predictors of the colony growth rate of both fungal species in black peppers. Temperature optimum (28-33°C) and a_w optimum (0.93-0.99) were estimated by multi-factorial cardinal model for both species in peppers. Following the growth study, production of aflatoxins, STERIG and OMST was analyzed using LC-MS/MS. Absence or very small production of mycotoxins in peppers following heavy mould growth shows that there seem to be a significant role of pepper constituents in interfering the mycotoxins biosynthesis at certain levels, with no or marginal influence on fungal growth and sporulation. The predictive growth models developed in this study could serve as a reliable tool in prevention of mould growth which could eventually control the spice spoilage and accumulation of mycotoxins in black pepper.

Chapter 9 describes the role of black pepper extract in growth and secondary metabolite production (aflatoxins, STERIG and OMST) of *A. flavus* and *A. parasiticus* isolates. Production of mycotoxins was heavily influenced by the concentration of the pepper extract applied (11-2660 ppm) while marginal effect on growth rate and sporulation was observed. At high concentrations of pepper extract >665 ppm none of these metabolites were produced by both species. Pepper extract concentration of 665 ppm could be suggested as the "minimum inhibitory concentration" for mycotoxins inhibition for *A. parasiticus* and 333 ppm for *A. flavus*. However, growth rate reduced only by 37% for *A. parasiticus* and 32-52% for *A. flavus* isolates at these concentrations. Potential application of pepper extract as a mycotoxin mitigation strategy in agricultural commodities either in pre- or post-harvest could be proposed and merits further research .

Chapter 10 presents the general conclusions, formulated future perspectives from overall findings of this PhD research, as well as emerging routes for further research and recommendations for the Sri Lankan government and food safety authorities on mycotoxin control.

SAMENVATTING

Sri Lanka, "de parel van de Indische Oceaan" in Zuid Azië is ook gekend als "Specerijen eilend", waar dure specerijen worden geproduceerd, geconsumeerd en geëxporteerd. Specerijen zijn gekend voor hun aroma, kleur, smaakeigenschappen in diverse keukens en ook wel voor hun therapeutische eigenschappen. De kwaliteit van de specerijen is vaak niet goed. Een ontwikkelingsland zoals Sri Lanka heeft zijn beperking in de productie van hoogwaardige specerijen voor de lokale consumptie en tracht ook te voldoen aan de vereisten die worden opgelegd door de importerende landen zoals Europa en US. Daarnaast heeft het eiland ook een tropisch klimaat, die de groei van specerijen zal stimuleren maar ook goed is voor ontwikkeling van schimmels en mycotoxinecontaminatie op het veld alsook tijdens naoogstactiviteiten zoals drogen, sorteren en verpakken. Mycotoxinen zijn secundaire metabolieten geproduceerd door schimmels. Net zoals andere levensmiddelen, kunnen ook specerijen zijn geïnfecteerd door schimmels en mycotoxinen bevatten. Dit onderzoekswerk is dan ook uitgevoerd om de actuele situatie in schimmel en mycotoxinecontaminatie van specerijen aanwezig op de Sri Lankaanse markt en een kwantitatieve risicobeoordeling van deze mycotoxinen uit te voeren, gezien de weinige informatie die aanwezig is op het eiland. In deze studie, werden chili peper (*Capsicum annum* L.) en peper (*Piper nigrum* L.) geselecteerd omdat dit de twee meest belangrijke specerijen op de wereld zijn inzake handel en consumptie.

Hoofdstuk 1 bevat een literatuurstudie. De resultaten uit het onderzoek zijn terug te vinden in hoofdstuk 2 tot en met 9, die hebben bijgedragen tot acht wetenschappelijke publicaties.

In **hoofdstuk 1** is achtergrondinformatie betreffende specerijen, chili, peper, hun gebruik, productie- en handelsdata weergegeven. Een gedetailleerde beschrijving van de chemische structuren, geassocieerde schimmelsoorten, hun aanwezigheid en toxiciteit van bepaalde belangrijke mycotoxinen is ook opgenomen. Daarnaast zijn de analytische detectietechnieken kort samengevat. De principes van risicoblootstelling met focus op mycotoxinen zijn uitgelegd. Finaal wordt de toepassing van voorspellend modelleren toegepast voor schimmelgroei, zogenaamde 'voorspellende mycologie' geïntroduceerd, de concepten en geschiktheid van verschillende wiskundige modellen wordt besproken.

Hoofdstuk 2 beschrijft een betrouwbare en snelle detectie gebaseerd op een QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe) extractieprocedure voor de bepaling van verschillende mycotoxinen aanwezig in specerijen, chili, zwarte en witte peper. High-performance liquid chromatography tandem massa spectrometrie (HPLC-MS/MS) was vervolgens gebruikt voor de kwantificatie en bevestiging van zeventien chemisch verschillende mycotoxinen. De mycotoxinen werden geëxtraheerd uit de gehydrateerde specerijen gebruikmakend van aangezuurd acetonitrile, gevolgd door een partitionering met NaCl en $MgSO_4$, hierdoor werd een 'dispersieve-vaste fase

extractie' bekomen. 'Electrospray ionizatie' met positieve mode werd gebruikt voor de simultane detectie van alle mycotoxinen in eenzelfde run van 20 min. Multiple reactie monitoring mode, waarbij tenminste twee gefragmenteerde ionen per analyte werden gebruikt, werd toegepast. Recoveries (75 tot 117%) waren in overeenstemming met de performantie criteria vereist door de Europese Commissie (EC, 401/2006). De kwantificatielimiet (LOQ) varieerde van 2,3 tot 146 µg/kg. De LOQ voldoet aan de maximum waarden van de twee wettelijk vastgelegde mycotoxinen namelijk, aflatoxine en ochratoxine A (OTA) in specerijen en dus kan de detectiemethode worden gebruikt voor de wettelijke bepalingen opgelegd door de Europese Commissie. De gevalideerde methode werd finaal gebruikt voor de screening van mycotoxinen in chili en peper stalen die werden verzameld in Sri Lanka zoals beschreven in Hoofdstuk 3 en 4.

Hoofdstuk 3 beschrijft de karakterisatie van de toxigene schimmels en de aanwezigheid van verschillende mycotoxinen in de Sri Lankaanse peperstalen. Karakterisatie van de schimmels werd uitgevoerd via *Aspergillus flavus* en *parasiticus* agar (AFPA) en ook via malt extract agar (MEA) voor 77 stalen zwarte peper en 11 stalen witte peper. *Aspergillus flavus, Aspergillus parasiticus*, *Aspergillus niger* en *Penicillium* spp. waren de meest voorkomende schimmels. In totaal, 73% van de zwarte peper en 64% van de witte peperstalen waren gecontamineerd met *Aspergillus flavus* en/of *Aspergillus parasiticus* (AfAp). De frequentie van voorkomen van *A. niger* in zwarte peper was 62% met tellingen tot $1.3x10^3$ kve/g. *Penicillium* spp. werd in respectievelijk 61% en 55% van de zwarte peper en de witte peperstalen teruggevonden. Andere *Aspergillus* spp., gevonden op de peperstalen zijn *Aspergillus terrus*, *Aspergillus tamarii*, *Aspergillus candidus*, *Aspergillus penicilloides*, *Aspergillus sydowii* en *Aspergillus fumigatus*. Schimmeltellingen op zwarte peper (10^2-10^4 kve/g) waren significant hoger dan deze teruggevonden op witte peper ($<10^2$ kve/g). Schimmelcontaminatie was ook opgevolgd tijdens de verschillende productiestappen, vormen van peper (vb. gemalen versus peperbolletjes en ook de regio's waar de stalen aangekocht werden.

Naast de aanwezigheid van de "klassieke mycotoxinen" op de specerijen, zijnde aflatoxine (<LOQ-18 µg/kg) en OTA (<LOQ-79 µg/kg), andere toxinen zoals fumonisine B1 (FB1; <LOQ-135 µg/kg), sterigmatocystine (STERIG; <LOQ-49 µg/kg) en citrinine (CIT; <LOQ-112 µg/kg) werden gedetecteerd in de stalen zwarte peper (n=82). STERIG werd zeer frequent gedetecteerd (44%). In totaal, 63% van de aflatoxine B1 (AFB1) positieve stalen overschreden zelfs de EU limiet van 5 µg/kg. Meer dan 63% van de zwarte peper stalen waren gecontamineerd met tenminste één mycotoxine en 12% had meer dan twee toxines. Mycotoxinecontaminatie in witte peper (n=11) was significant lager dan in zwarte pepper. Uiteindelijke werden ook 27 peperstalen gekocht op de Belgische markt en geanalyseerd, waarbij ook verscheidene mycotoxinen warden teruggevonden.

Hoofdstuk 4 beschrijft het samen voorkomen van meerdere mycotoxinen in gedroogde chili stalen (n=86) verzameld in Sri Lanka. Naast aflatoxinen (<LOQ-687 µg/kg) en OTA (<LOQ-282 µg/kg), werden nog andere mycotoxinen teruggevonden zijnde STERIG (<LOQ-32 µg/kg), fumonisine B2 (FB2; <LOQ-87 µg/kg) en CIT (<LOQ-2.1 mg/kg). AFB1 was de meest voorkomende mycotoxinen waarmee bijna 77% van de stalen was besmet. Opmerkelijk, 67% van de stalen overschreden de EU limiet van 5 µg/kg voor AFB1 en 44% overschreden de EU limiet van 10 µg/kg voor totale aflatoxines. Terwijl OTA werd teruggevonden in 41% van de stalen waarvan ook 38% van de stalen waren gecontamineerd met STERIG. In het totaal, 87% van de stalen, was gecontamineerd met minstens één mycotoxine. Eén derde van de chili stalen waren gecontamineerd met meer dan drie verschillende toxines. Samen voorkomen van verschillende mycotoxinen, AFB1-OTA (36%), AFB1-STERIG (28%), OTA-AFB1-STERIG (17%) en AFB1-FB2 (14%) werd gevonden in verschillende vormen van gedroogde chili. Hogere frequentie van samen voorkomen van mycotoxinen werd teruggevonden in verder verwerkte chiliproducten zoals vlokken en poeder en zou kunnen te wijten zijn aan frauduleus gebruik van lage kwaliteit aan grondstof. Om te kunnen vergelijken werden ook nog stalen chili aangekocht op de Belgische markt (n=35).

Hoofdstuk 5 omvat een kwantitatieve risicobeoordeling (deterministisch en probabilistisch) van de mycotoxinen via de consumptie van de chili en zwarte peper in Sri Lanka. Een voedselinname bevraging werd uitgevoerd om data te verzamelen betreffende de consumptie van deze specerijen in de huishoudens in Sri Lanka, in het Noorden en het Zuiden van het eiland (n=249). De gemiddelde blootstelling voor AFB1 in het Noorden (3,49 ng/kg LG/dag) en het Zuiden (2,13 ng/kg LG/dag) overschreden de voorgestelde tolereerbare inname (1 ng/kg LG/dag) door chili consumptie in het lagere band scenario (deterministische berekening) terwijl de blootstelling voor OTA laag was. De blootstelling voor andere mycotoxinen FB1, FB2, STERIG en CIT werd ook berekend. 'Margin of exposure' berekeningen toonden aan dat de gemiddelde blootstelling voor AFB1 veel lager waren voor chili (45-78) dan voor peper (2315-10857). Meer, het hepato cellular carcinoma (HCC) risico geassocieerd met de gemiddelde blootstelling voor AFB1 voor chili consumptie in lager band scenario per jaar werd berekend op 0,046 en 0,028 HCC gevallen/jaar/100,000 gebaseerd op de consumptie in het Noorden en in het Zuiden respectievelijk. AFB1 blootstelling via chili moet daarom beschouwd worden als een belangrijke bezorgdheid voor de publieke gezondheid in Sri Lanka zowel door de hoge mycotoxine concentraties als door de hoge consumptie van deze specerijen.

Hoofdstuk 6 toont de sorptie-isothermen die zijn opgesteld voor zwarte peperbollen bij 22, 30 en 37°C gebruik makend van de standaard statische gravimetrische methode. De sorptie-isothermen vertoonden een type III gedrag volgens Brunauer-Emmett-Teller (BET) classificatie. Het evenwichtsvochtgehalte (EMC) daalde met stijgende temperatuur bij een specifieke wateractiviteit.

Hysteresis werd opgemerkt over de ganse range aan wateractiviteit die werd onderzocht bij 0° en 37°C. Maar bij 22°C, een intersectie van de curves werd gedetecteerd bij wateractiviteiten dichter bij 0,75. Elf beschreven sorptie-isothermen werden gefit aan de experimentele data. De Guggenheim-Anderson-de Boer (GAB) en Peleg modellen sloten het nauwst aan bij de data. De Oswin, geadapteerde Oswin, geadapteerde Mizrahi, dubbel log polynomiaal modellen beschreven de data adequaat. Het monolaag vochtgehalte (M_0), berekend gebruik makend van de GAB modellen waren 3,49-4,78% voor de adsorptie en 4,36-4,67% voor desorptie. De maximum isosterische hittesorptie was 28,1 en 73,3 kJ/mol voor adsorptie en desorptie, respectievelijk en dit was bij 4% EMC. De Gibbs vrije energie wisseling (-ΔG) varieerde tussen 100,6-9370,6 J/mol afhankelijk van de temperatuur en EMC (1-40%). De ontwikkelde sorptie-isothermen kunnen gebruikt worden tijdens de industriële drogingsprocessen, verwerking en bewaring van volledige bollen zwarte peper.

Hoofdstuk 7 beschrijft verder de toxigeniteit van *A. parasiticus* en *A. flavus* isolaten afkomstig van de Sri Lankaanse peperstalen. Een LC-MS/MS methode werd ontwikkeld voor twintig secundaire metabolieten die mogelijks geproduceerd werden door verschillende schimmels (*Aspergillus, Penicillium, Fusarium* en *Alternaria* spp.) op malt extract agar, te kwantificeren. Het mycotoxigenisch potentieel van *Aspergillus flavus* (n=11) en *A. parasiticus* (n=6) stammen geïsoleerd van zwarte peper werd gevolgd tijdens hun groei op malt extract agar bij 22, 30 en 37°C gebruik makend van de ontwikkelde detectiemethode. Al de *A. flavus* isolaten produceerden AFB1 en O-methyl sterigmatocystine (OMST) terwijl 91% aflatoxine B2 (AFB2) produceerden en 82% van de stammen ook STERIG bij 30°C. Behalve één stam, produceerden alle *A. parasiticus* isolaten de vier aflatoxines, STERIG en OMST bij 30°C, maar de productie van aflatoxine G was veel lager. De volgende trend in mycotoxine productie kan worden vastgesteld: 30°C>22°C>37°C, terwijl de groeisnelheid een andere trend toonde, 30°C>37°C>22°C voor alle species. Meer, interessante correlaties konden worden teruggevonden tussen de schimmelsoorten en de mycotoxine productie.

Hoofdstuk 8 omvat de discussie betreffende groei en mycotoxine productie van één *A. parasiticus* en drie *A flavus* isolaten in volledige zwarte peperbolletjes gebruik makend van een volledige factoriële proefopzet met zeven verschillende niveau's in wateractiviteit (a_w) (0,826-0,984) en drie temperaturen (22, 30 en 37°C) om voorspellende groeimodellen te kunnen opstellen. Groeisnelheid en lag fases werden geschat door gebruik te maken van lineaire regressie. Verschillende secundaire kinetische modellen werden opgesteld om de radiale groei van de schimmels als functie van individueel of gecombineerd effect van a_w en temperatuur. Tijdens de evaluatie van de statistische indices, werd het 'Rosso square root cardinal model' aanbevolen voor de beschrijving van het individueel effect van de a_w terwijl het 'extended Gibson models' gebruikt werden voor de beste fit

om het gecombineerde effect van a_w-temperatuur op de groei van beiden *A. flavus* en *A. parasiticus* species op de peperbolletjes. De biasfactoren (0,70-1,01), accuriteitsfactoren (1,01-1,41) en root mean square error (0,019-0,280) toonden aan dat de gefitte modellen vrij conservatief de koloniegroeisnelheid voorspellen van beide schimmels. Temperatuur optimum (28-33°C) en a_w optimum (0,93-0,99) werden geschat via het 'multi-factorial cardinal model' voor beide soorten schimmel op de peper. Na de groeistudie, werd ook de productie van de mycotoxinen aflatoxine, STERIG en OMST gebruik makend van LC-MS/MS detectie. Afwezigheid of een zeer lage productie van de mycotoxinen op de peperbolletjes na de sterke schimmelgroei toont aan dat er een significante interferentie is van de pepersamenstelling op de mycotoxine biosynthese maar geen of marginale invloed op de schimmelgroei of-sporulatie. De voorspellende groeimodellen in deze studie kunnen dienen als een betrouwbare tool om de schimmelgroei in te schatten bijvoorbeeld tijdens de bewaring van peperbollen.

Hoofdstuk 9 beschrijft de rol van extract gemaakt van zwarte peper op de groei en de mycotoxine productie (aflatoxine, STERIG en OMST) van *A. flavus* en *A. parasiticus* isolaten. Productie van mycotoxinen was sterk beïnvloed door de toegepaste concentratie van het peperextract (11-2660 ppm) terwijl slechts een marginaal effect op de groeisnelheid en de sporulatie werd teruggevonden. Bij hoge concentraties van het peperextract >665 ppm werden geen van de mycotoxinen geproduceerd door de beide schimmels. Daardoor kan de extractconcentratie van 665 ppm worden voorgesteld als de "minimale inhibitieconcentratie" voor *A. parasiticus* en 333 ppm voor *A. flavus*. Echter de groeisnelheid werd slechts voor 37% gereduceerd bij *A. parasiticus* en 32-52% voor *A. flavus* isolaten bij deze concentraties. Potentiële toepassing van het peperextract als een interventiestrategie ter voorkoming van mycotoxine productie toegepast op plantaardige producten zowel voor- als naoogst kan worden voorgesteld maar behoeft wel nog verder onderzoek.

Hoofdstuk 10 stelt de algemene conclusies voor, de toekomstperspectieven van dit onderzoekswerk, maar ook de aanbevelingen voor de diverse stakeholders in Sri Lanka om tot een bewaking te komen van de voedselketen inzake mycotoxinen in specerijen.

CHAPTER

1

LITERATURE REVIEW

CHAPTER 1: LITERATURE REVIEW

1.1. INTRODUCTION TO SPICES

Spices have played a dramatic role in civilization and in the history of nations. The delightful flavour and pungency of spices make them indispensable in the preparation of pleasant dishes. Spices as sources of natural colours, flavours and aroma present welcome opportunities in the international market. The nutritional, antioxidant, antimicrobial and therapeutic properties of spices have widespread applications (Peter, 2006). The major markets in the global spice trade are the United States of America (USA), the European Union (EU), Japan, Singapore, Saudi Arabia and Malaysia. The principal supplying countries are China, India, Madagascar, Indonesia, Vietnam, Brazil, Spain, Guatemala and Sri Lanka (Zachariah and Parthasarathy, 2008; FAO, 2011). The spice exports are subject to strict quality standards for food safety set by the American Spice Trade Association (ASTA) and the European Spice Association (ESA). The two most important spices of world trade, chilli and pepper, are briefly described hereunder.

1.1.1. Chilli

Chilli belongs to the genus *Capsicum*, which consists 22 wild species. Among them, *Capsicum annum* L. is a widely cultivated species in tropical, subtropical and temperate countries (Beletti et al., 1998). Globally, there are more than 3000 known varieties of chilli peppers that differ in shape, color, size, flavor and degree of pungency, thus the use of the term "chilli pepper" is often being confused. Pepper, chili, chile, chilli, paprika, picante, cayenne and capsicum are interchangeably used for spices of the genus *Capsicum* (Zachariah and Gobinath, 2008). Chilli is the word for hot chilli peppers used in Asia, while the term capsicum/paprika is used for the non-hot sweet bell peppers. The Food and Agriculture Organization (FAO) also refers chilli to the hot varieties. Chillies dominate the culinary flavors in many cultures; Indians, Sri Lankans, Southeast Asians, Latin Americans, North Africans and the Caribbean islanders. Sri Lankans and South Indians use whole cut chillies abundantly in snacks, chutneys and curries (Raghavan, 2000). India is the largest producer and exporter of chilli while the USA is the main importer (FAO, 2011). Top chilli producing, importing and exporting countries (% quantity MT) are shown in Fig. 1-1.

Chilli is often used as a food ingredient to impart hot sensation, flavor, color and visual appeal to a great variety of cuisines around the world. Chilli pungency is produced by the capsaicinoids, a group of alkaloid compounds that are found only in the genus *Capsicum*, while approximately 20 carotenoids contribute to the red colour of chilli powder (Zachariah and Gobinath, 2008). Hot chillies are pickled or pureed and are used as table condiments or sauces. The nutritive value of chilli is high and it is an excellent source of vitamins C (ascorbic acid), A, B-complex and E along with minerals like molybdenum, manganese, folate and potassium. Chilli contains seven

times more vitamin C than orange. Beta-carotenoids, vitamins C and A in chillies are powerful antioxidants that stabilize free radicals (Simmone et al., 1997).

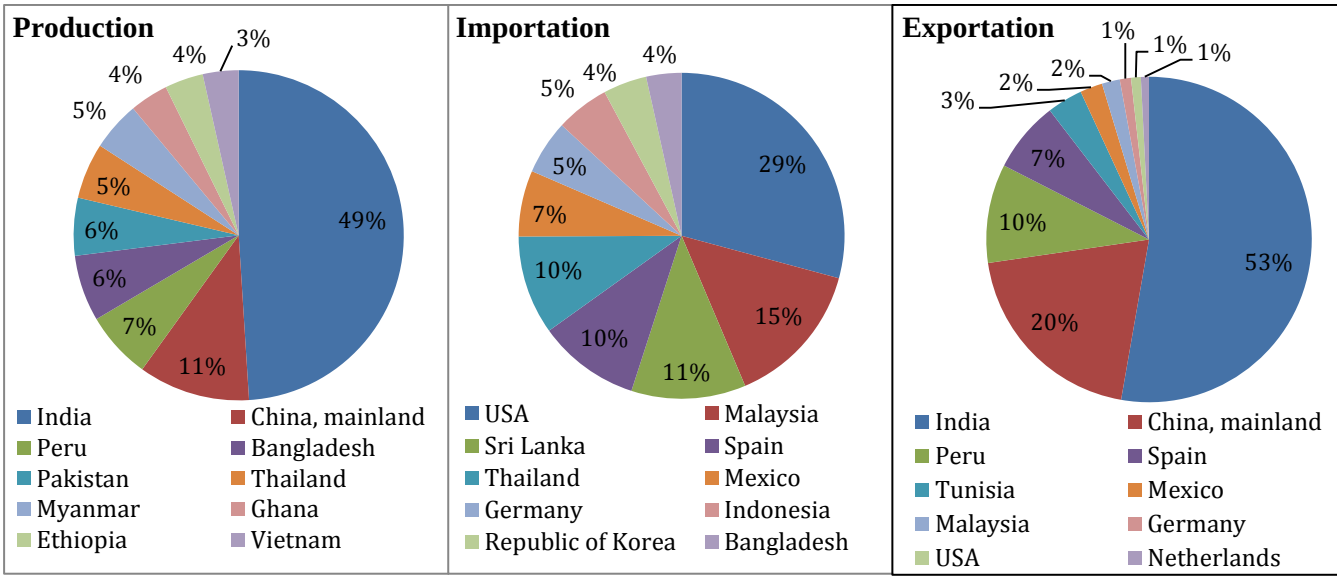


Fig. 1-1. Top chilli producing, importing and exporting countries (FAO, 2011, 2012)

Chillies have long been recognized by many cultures around the world also for their therapeutic qualities. They are believed to increase blood circulation, relieve rheumatic pain, treat mouth sores and infected wounds, reduce blood clots, aid digestion and are known to have anti-cancerous activity (Raghavan, 2000; Ravishankar et al., 2003; Zachariah and Gobinath, 2008).

1.1.1.1. Pre-harvest practices of chilli

Acccording to Department of Agriculture (DOA), Sri Lanka, well drained and fine soils with sufficient sunlight are essential factors for nursery preparation for chilli. Seeds are treated with fungicide, 80% captan. After sterilization of the nursery buds (burning/solarization/using chemicals), the seeds are sown in rows and watered daily. Chilli grows well in deep, loamy, fertile and well drained soils. Land is prepared by deep ploughing. Raised/sunken beds or ridges and furrows are prepared for the transplanting of the seedlings. In Sri Lanka chilli is cultivated in both dry (mid Mar to Aug) and wet (mid Sep to Feb) seasons. Fertilizer application (urea, triple super phosphate, muriate of potash) is generally more than the DOA recommendations. Very limited number of farmers apply more organic manures than the chemicals. Weedicide is applied during land preparation. Hand weeding is practiced after planting. Diseases reported in chilli are, damping off, Anthracnose, leaf spots, powdery mildew, foot rot, bacterial wilt and chilli leaf mosaic virus (DOA, Sri Lanka). Nursery sterilization, field level sanitation and use of fungicides are some of the adopted control measures. Chilli plants start to flower in about 75-80 days of sowing.

1.1.1.2. Post-harvest practices of chilli

First harvest of chilli pods are performed usually from 110 to 120 days of sowing. Well matured green pods are harvested for green chilli purpose; picked in the morning or in the evening. For dry chilli production, chilli pods are harvested mature when they start to turn into red in colour; more than 80% pods in red stage. The pods are hand-picked carefully in aluminum or plastic vessels or in jute bags. Under well managed cultivation practices, on average 10-12 picks can be done as green chilli or 7-9 picks as red chilli. Time of harvesting, number of picks and yield slightly varies with variety (DOA, Sri Lanka). Chillies on harvesting have a moisture content of 65-80% depending on whether partially dried on the plant or harvested while still succulent, this must be reduced to 10% to prepare dried spice.

Processing of dry chilli is done in very limited scale in Sri Lanka. Mature red chilli pods are heaped in floor or kept in gunny bags in room temperature for the development of red colour of the partial turned red pods. After harvesting, the chilli pods are spread in the cement floors/mats/tarpaulins or even directly in the soil in thin layers for sun drying. Pods are overturned timely to facilitate uniform drying. Good quality dry chillies are produced in 10-12 days after drying under sunlight (DOA, Sri Lanka). Well dried chillies are packed in gunny bags and stored at room temperature conditions. Most of the dry chillies consumed in Sri Lanka were imported from India or China and processed locally to produce chilli powders or crushed chilli.

1.1.2. Black Pepper

The black pepper of commerce is the matured dried berries (peppercorns) of the tropical, perennial plant *Piper nigrum* L., of the *Piperaceae* family. Black pepper has been widely used as spice since time immemorial. Black pepper is considered as the "king of spices" as it fetches the highest return from international trade (Srinivasan, 2007), and is also known as "Black Gold". Peppercorns are, by monetary value, the most widely traded spice in the world, accounting for about 20% of all spice imports and for about 35% of the world trade in spices (Zachariah and Parthasarathy, 2008). The top pepper producing, importing and exporting countries (% quantity MT) are shown in Fig. 1-2.

Black pepper holds a prominent position among spices as it has multiple functional uses, such as seasoning in vast food formulations, pharmaceuticals, preservatives and even in cosmetics (Butt et al., 2013; Singletary, 2010). The distinct biting quality of pepper is mainly due to the alkaloid piperine (5-9%) and its isomers that imparts sensational flavour and taste to food while the oleoresin containing essential oils (1-3%) impart aroma. Piperine is pharmacologically safe and also figures in the US Food and Drug Authority (FDA) list of 'Generally Regarded as Safe' (GRAS) compounds. Numerous other bioactive (pharmacological/medicinal) properties of pepper that could benefit human health include, antimicrobial, antioxidant, anticarcinogenic/antigenotoxic,

antidepressant, antidiarrheal, antiulcer, immunomodulatory, insecticidal and insulin resistant (Butt et al., 2013).

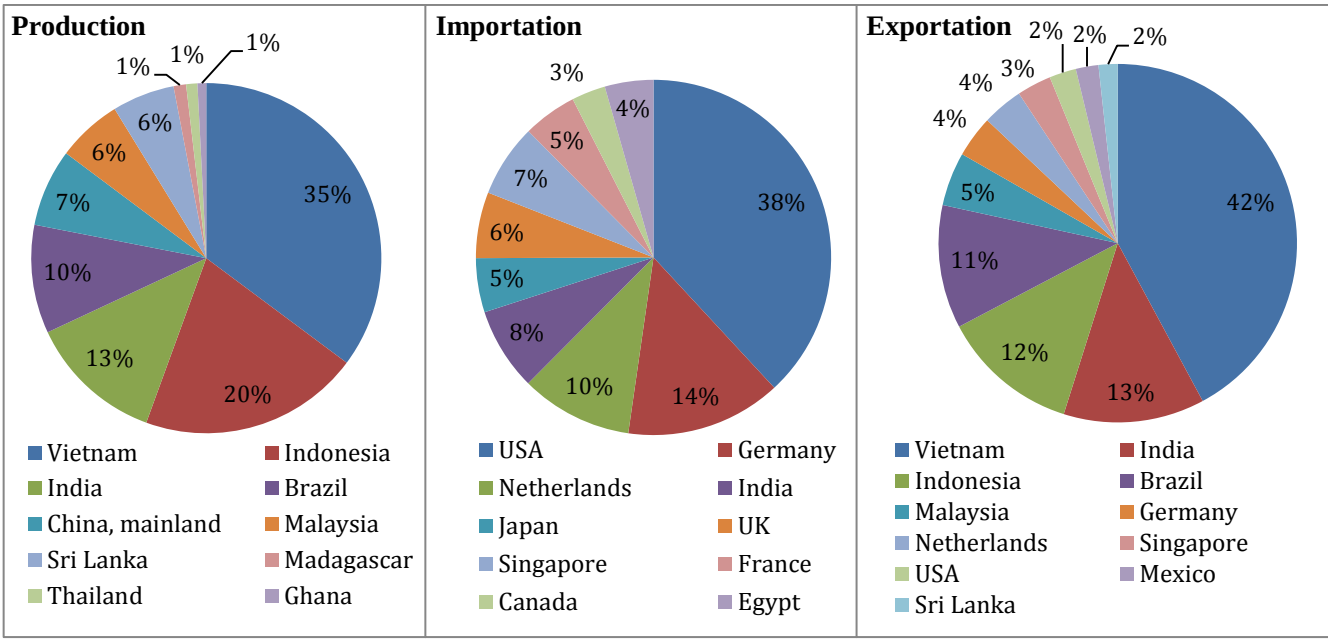


Fig. 1-2. Top pepper producing, importing and exporting countries (FAO, 2011, 2012)

(During the initial phase of this research work (2011), questionnaires (for farmers and processors) were prepared in order to collect pre- and post-harvest information on pepper. Some potential pepper farmers and processors from the pepper growing areas were identified with the help of Department of Export Agriculture (DEA), Sri Lanka and they have been interviewed. Hence, the information presented below on pre- and post-harvest practices of pepper were obtained using questionnaires and details gathered from DEA).

1.1.2.1. Pre-harvest practices of peppers

In Sri Lanka pepper is mainly cultivated in low, mid country, wet and intermediate agro-ecological zones. Although the origin of black pepper is believed to be Malabar Coast of India, Sri Lanka too is a home to a number of wild pepper types. High yielding pepper line called "Panniyur-1" from India and "Kuchin" from Malaysia was introduced in 1970s but MB21 and GK 49 are high yielding and superior quality local selections which are popular among black pepper cultivators. Total extent of pepper production in Sri Lanka is about 31,162 ha (DEA, 2011). Matale, Kandy, Kegalle, Badulla, Ratnapura, Monaragala and Kurunegala are the major pepper growing districts. Pepper grows best in well drained loamy soils rich in organic matter. Clay soil restricts root growth and creates moisture stress during short dry spells. Ill drained soil leads to many soil borne diseases. Pepper grows well in mid and upcountry areas with annual rain fall of not <1750 mm. Supplementary irrigation is necessary in areas with long dry periods. Sufficient rainfall is essential during flowering to facilitate pollination. Plants can tolerate 15-40ºC. Pepper is usually propagated vegetatively using stem cuttings; terminal stems, lateral branches and basal runners. Field planting is done at a spacing of 2.4m x 2.4m. After the land preparation planting pits of 45cm x 45cm x

45cm are made and filled with the mixture of top soil, cow dung or compost. Pepper vines are trained on live or dead supports. In Sri Lanka live supports are used, eg., *Glyricidia sepium*, *Erythrina indica*. After 3-5 years pepper vine grows to the top of the standard and makes a good canopy. At the height of 3.5-4.0 m pruning is done to maintain the height of the pepper plant and to make a good shape canopy. First dose of fertilizer is applied in 15-30 days of planting. Urea, rock phosphate and muriate of potash are applied. No major pests have so far been observed to attack peppers in Sri Lanka. Minor incidences of pepper thrips, shoot borers and sporadic occurrence of diseases like quick wilt, leaf rot, leaf blight (*Phytopthora palmivora*), yellowing (*Fusarium* spp.) and little leaf disease (virus) have been reported. Generally, 1% bordeux mixture or captan was used to control such disease.

1.1.2.2. Post-harvest practices of peppers

Pepper harvesting seasons vary from country to country, depending on climate. India, Sri Lanka, and Thailand have long harvesting period compared to the other producing nations. There are two harvesting seasons in Sri Lanka because of the bi-model rainfall distribution, with 70% is harvested during November-January (Yala) and remaining produce is harvested from May to July (Maha).

Black pepper: Major proportion (90%) of pepper harvested in Sri Lanka is processed as black pepper. The correct stage of harvesting for the preparation of black pepper is when few berries in the spikes begin to change from green to yellow colour (fully mature but unripe, 7-8 months of maturity). The berries at harvest contains 70-80% moisture. Harvesting is done manually, each spike is picked off carefully in gunny bags/plastic/aluminium vessels without damaging the tender branches or loosening the vines from the supports.

In Sri Lanka, the harvested spikes are heaped for 2-3 days and thereafter, berries are separated/detached from the spikes by trampling underfoot/crushing by hand or beating with sticks. Mechanical threshers are also available but limited. The larger pieces of the spikes are removed by hand and the smaller pieces are removed by winnowing. After this, washing of the berries is done by hand and drained-off. Sun drying is most commonly used for processing as this is the cheapest method and most practical to reduce moisture content. The berries are spread in thin layer on the cement floor as soon as they are separated from the spikes; mostly practiced by the large scale farmers. But the small scale farmers geneally dry pepper berries on poly sheets or mats laid along the road sides; potential microbial contamination if allowed to contact soil. During the drying process browning enzymes from the cell walls of the berries are released. The berries are periodically turned over (3/4 hours) to facilitate uniform drying. However, the sudden rain shower in the pepper growing areas is a hindrance for uniform drying. Depending on the rain and the overcast conditions the drying process takes around 3-4 days with blanching. Blanching (immersing

in hot water at 80-90°C for 3 min) is recommended by DEA to assure the attractive black colour, but seldom practised by small scale farmers. Without blanching the traditional sun drying process takes around 6-7 days. Use of advance technology for drying is not possible by the small scale pepper producers as they have no access to high tech driers; hence, use of clean concrete floor is encouraged. Few large scale pepper processors are using such advance dryers. Some farmers transport the pepper to speed-up the drying process. The completion of drying is identified by the experienced farmers by the black wrinkled appearance of skin and feel of dryness with hands; they are also aware about the final weight which reduces the return. Removing the dust, dirt and grading are usually done manually with winnowing baskets and sieves. Considering the secondary processing, motorized mills or mechanical grinders are used in the production of ground pepper or crushed pepper by few large scale pepper processing industries.

White pepper: The pepper berries are harvested at 8-9 months of maturity when the berries are fully ripened and turned orange-red outer pericarp to produce white peppers. The collected berries are collected in gunny bags/vessels and steeped in flowing water for 7-10 days. This process helps to soften the seed coat ("retting"). The seed coat is then removed by rubbing them by hands in a bucket of water or on a wire mesh or using mechanical decorticator. The seeds are then washed with fresh water and dried in thin layer for 3-4 days in sun. The seeds are further cleaned manually by winnowing and polished by rubbing in cloth.

Storage: Traditionally, fully dried pepper is stored in jute or poly sacks sufficiently for longer time at room temperature conditions. Sacks are stacked in close fitted store rooms above the wooden planks as dunnage placed on floors. The produce is checked intermittently for moisture absorption; then dried again in sun.

1.2. INTRODUCTION TO MOULDS AND MYCOTOXINS

Mycotoxins are small (MW~700 Da) toxic chemical products formed as secondary metabolites (>500) by few fungal species, mostly by saphrophytic moulds (Pitt and Hocking, 2009; Turner et al., 2009). These secondary metabolites can be important in host-pathogen relationship, with some providing protection against environmental stresses and as well as fungivory (Rohlfs et al., 2007). Agricultural produce can be contaminated with mycotoxins before harvest, during the time between harvesting and drying, processing and in storage. Spices are largely produced in countries where tropical climates (high ranges of temperature, humidity and rainfall) are favorable to mould infestation and thus mycotoxin contamination. Furthermore they are usually dried on the ground in open air in poor hygienic conditions that promote the growth of moulds and production of mycotoxins (Martins et al., 2001; Santos et al., 2011). Among spices, chilli and peppers have been reported to be frequently contaminated with aflatoxins and ochratoxins (Fazekas et al., 2005; Santos

et al., 2010; Jalili et al., 2010; Yogendrarajah et al., 2014a and 2014b). Major classes of mycotoxins (probable to occur in spices), more specifically aflatoxins, sterigmatocystin, ochratoxins, trichothecenes, fumonisins, zearalenone, citrinin and alternaria toxins are briefly discussed in this section.

1.2.1. Aflatoxins

Aflatoxins are a group of closely related and widely researched mycotoxins produced by *Aspergillus* spp. Aflatoxins can be found in the field before harvest and contamination can increase during post-harvest activities, e.g., crop drying or in storage. However, aflatoxins also can contaminate stored products in the absence of field contamination. Only few species of fungi are known to produce aflatoxins and they all belong to *Aspergillus* section *flavi*. These species are *Aspergillus flavus*, *Aspergillus parasiticus*, *Aspergillus nomius*, *Aspergillus pseudotamarii*, *Aspergillus bombycis* and *Aspergillus ochraceoroseus* (Bennet and Klich, 2003; Peterson et al., 2001; Varga et al., 2009). Among them, *A. flavus* and *A. parasiticus* are economically important and they have overlapping niches while the others are encountered less frequently.

In nature, *A. flavus* is one of the most abundant and widely distributed soil-borne moulds found on earth (Yu and Keller, 2005). However, there are qualitative and quantitative differences in the toxigenic abilities between different strains within each aflatoxigenic species. It is said that only half of the *A. flavus* strains can produce more than 10^6 µg/kg aflatoxins (Klich et al., 1988). Generally, *A. flavus* produces only B aflatoxins (AFB1 and AFB2) while *A. parasiticus* produces both B and G aflatoxins (AFG1 and AFG2). *A. flavus* produces asexual spores, conidia and the asexual fruiting body, sclerotia, which is a resistant structure that enables the strain to survive under harsh conditions (Amaike and Keller, 2011). A schematic diagram of *A. flavus* conidiophores is shown in Fig. 1-3. *A. flavus* also causes mycoses (infection with fungus as opposed to diseases caused by the consumption of fungal toxins which are broadly known as mycotoxicoses) in humans and animals. *A. flavus* is unique in that it is an opportunistic pathogen of both plants and animals (Amare and Keller, 2014).

Aflatoxins are difuranocoumarin derivatives produced by a polyketide pathway by many strains of *A. flavus* and *A. parasiticus*. They have a polycyclic structure derived from a coumarin nucleus attached to a bifuran system. B type aflatoxins are attached to a pentanone, while G types are attached to a 6-membered lactone (Fig. 1-4).

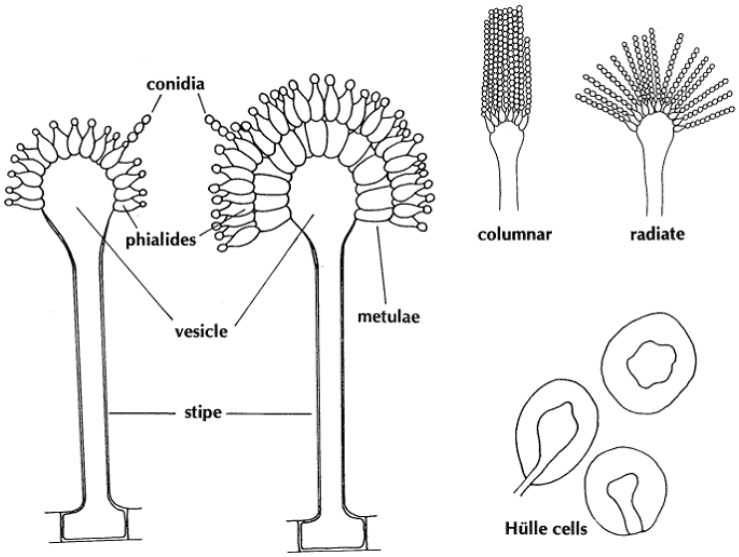
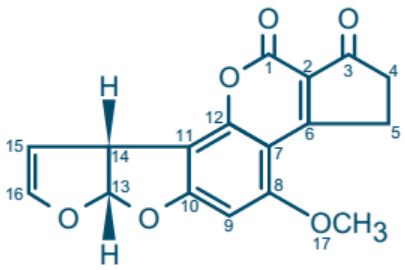


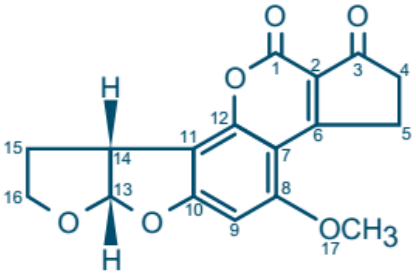
Fig. 1-3. Conidiophores of *Aspergillus flavus* showing mono- (columnar and radiate) and bi-seriate conidial heads (Samson et al., 2004).

Cereals, figs, corn, peanuts, cottonseed nuts, tobacco, spices, cheese, Brazil nuts, copra, almonds and a long list of other food and feed commodities are commonly contaminated with aflatoxins (Bennet and Klich, 2003). Aflatoxins may be metabolized and therefore occur in animal products as well. These include aflatoxins M1 and M2 which are oxidative metabolic products of aflatoxins B1 and B2 and usually excreted in milk (both animal and human), urine and faeces (Peraica et al., 1999).

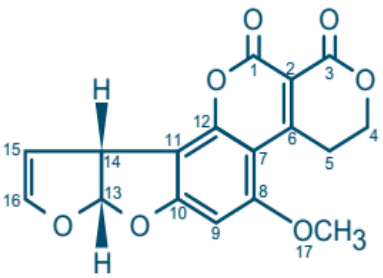
AFB1



AFB2



AFG1



AFG2

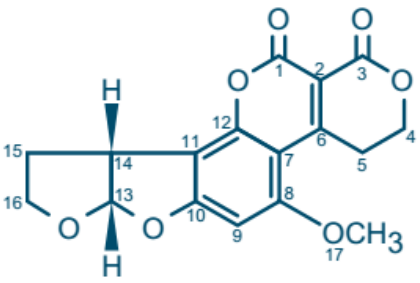


Fig. 1-4. Chemical structures of aflatoxins: AFB1, AFB2, AFG1 and AFG2

Among aflatoxins, AFB1 is the most toxic and most potent hepatocarcinogenic natural compound ever characterized (Yu and Keller, 2005). AFB1 is considered as a risk factor in the development of hepatocellular carcinoma (HCC) in Africa and Asia (Scholl and Groopman, 2008). It has been

classified as a group 1 human carcinogen (IARC, 1993a). AFB1 is metabolized in the liver to AFB1 8,9-*exo*-epoxide that binds to DNA to produce AFB1-N7-guanine adduct (Wild, 2002). AFB1 is more mutagenic and carcinogenic than AFG1, reflecting that the AFB1 8,9-*exo*-epoxide intercalates more readily into DNA than AFG1. AFB2 and AFG2 are far less biologically active (Wild and Gong, 2010). Moreover, aflatoxins are also immuno suppressive (Turner et al., 2003) and cause growth impairment in children (Turner et al., 2007; Gong et al., 2002). In an acute aflatoxin outbreak in Kenya (2004), 125 people died with 317 reported illnesses due to contaminated maize consumption (Lewis et al., 2005). Williams et al. (2004) have estimated that 4.5 billion of the world's population is exposed to aflatoxins.

1.2.1.1. Aflatoxins biosynthesis and regulation

Aflatoxins biosynthesis is the best characterized of any fungal secondary metabolite (Klich et al., 2007). The whole genome sequences of *A. flavus* has been released and reveals 55 secondary metabolite clusters that are regulated by different environmental regimes. The estimated genome size of *A. flavus* is 36.8 Mb with approximately 12,000 predicted functional genes (Amaike and Keller, 2014). To date only three secondary metabolite clusters have been characterized in *A. flavus*, aflatoxin, cyclopiazonic acid (CPA) and aflatrem. Similar to many secondary metabolites, genes of aflatoxin biosynthesis are also clustered (Fig. 1-5). The complete cluster has been sequenced and annotated (Yu et al., 2004). The aflatoxin cluster is composed of approximately 30 different genes and is located near the telomere of chromosome 3, the telomere distal from the CPA cluster. *aflA*, *aflB*, (fatty acid synthase) and *aflC* (polyketide synthase) are required to synthesize the first stable aflatoxin precursor, norsolorinic acid (NOR). NOR is further synthesized to sterigmatocystin and then to aflatoxin (Yu et al., 2004).

The regulation of aflatoxin biosynthesis has been studied intensively (Bhatnagar et al., 2003 & 2006; Price and Payne, 2005; Georgianna and Payne, 2009; Yu, 2012; Cary et al., 2014) to facilitate development of control strategies by understanding their biosynthesis. Regulation of aflatoxin gene expression occurs at multiple levels and by multiple regulatory components. There are genetic factors, biotic and abiotic elements that affect aflatoxin formation. As mentioned, the aflatoxin pathway genes are found to be clustered in the genome of *A. flavus* and *A. parasiticus*. These genes are expressed concurrently except for the regulatory gene *aflR*. In this gene cluster, a positive-acting regulatory gene, *aflR*, is located in the middle of the gene cluster. Adjacent to *aflR* a divergently transcribed gene, *aflS* (*aflJ*), was also found to be involved in the regulation of transcription (Meyers et al., 1998). Deletion of *aflR* in *A. parasiticus* abolishes the expression of other aflatoxin pathway genes (Cary et al., 2000).

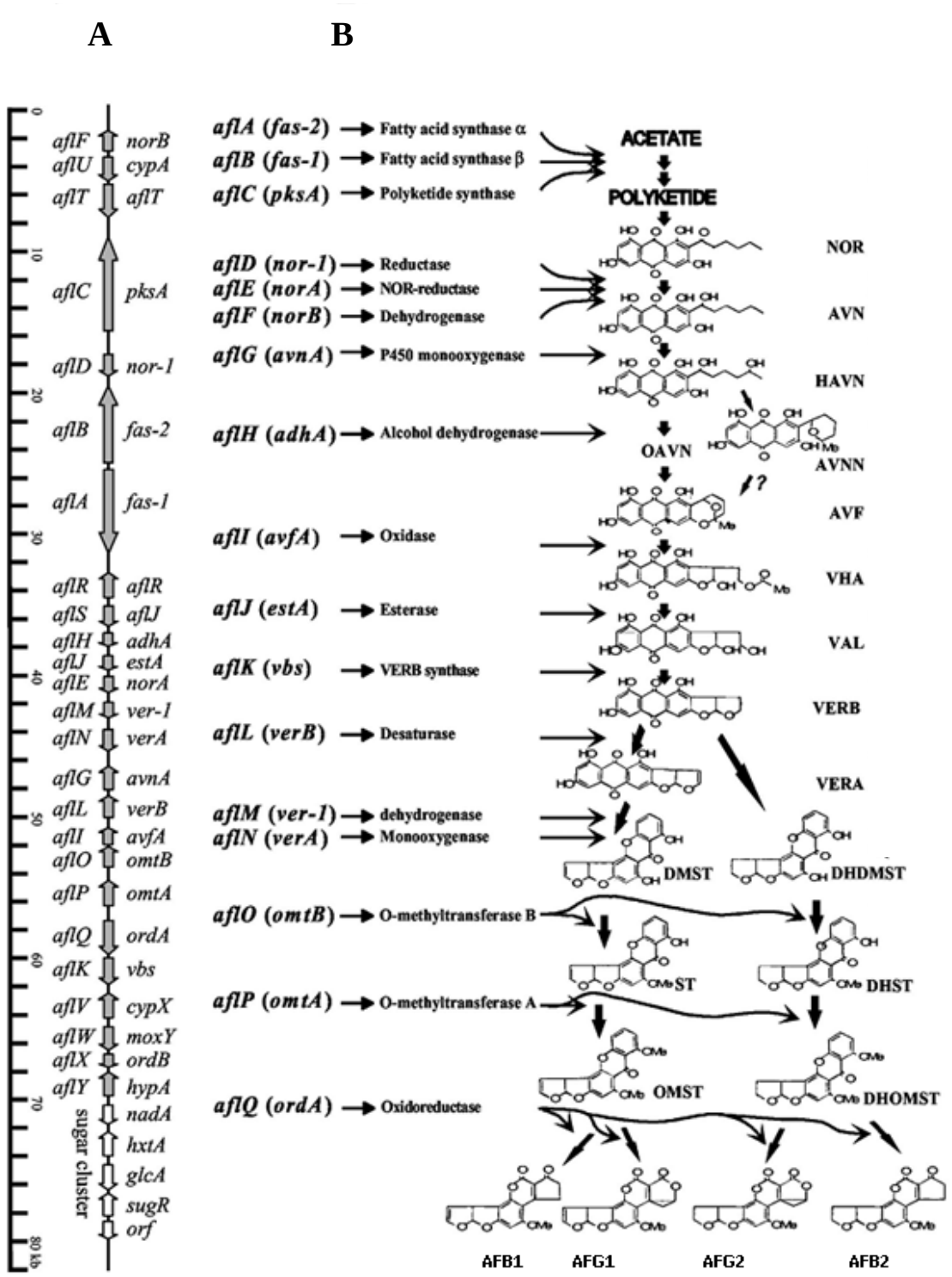


Fig. 1-5. Clustered genes (A) and the aflatoxin biosynthetic pathway (B). The corresponding genes and their enzymes involved in each bioconversion step are shown in panel A. The vertical line represents the 82-kb aflatoxin biosynthetic pathway gene cluster and sugar utilization gene cluster in A. Parasiticus and A.flavus. The new gene names are given on the left of the vertical line and the old gene names are given on the right. Arrows along the vertical line indicate the direction of gene transcription. The ruler at far left indicates the relative sizes of these genes in kilobases. Arrows in panel B indicate the connections from the genes to the enzymes they encode, from the enzymes to the bioconversion steps they are involved in, and from the intermediates to the products in the aflatoxin bioconversion steps. Abbreviations: NOR, norsolorinic acid; AVN, averantin; HAVN, 5 -hydroxyaverantin; OAVN, oxoaverantin; AVNN, averufanin; AVF, averufin; VHA, versiconal hemiacetal acetate; VAL, versiconal; VERB, versicolorin B; VERA, versicolorin A; DMST, demethylsterigmatocystin; DHDMST, dihydrodemethylsterigmatocystin; ST, sterigmatocystin; DHST, dihydrosterigmatocystin; OMST,O-methylsterigmatocystin; DHOMST, dihydro-O-methylsterigmatocystin; AFB1, aflatoxin B1; AFB2, aflatoxin B2; AFG1, aflatoxin G1; AFG2, aflatoxin G2 (Yu et al., 2004).

Overexpression of *aflR* in *A. flavus* up-regulates aflatoxin pathway gene transcription and aflatoxin accumulation (Flaherty & Payne, 1997) in a fashion similar to that reported for *A. parasiticus* (Chang et al., 1995). These results demonstrate that *aflR* is specifically involved in the regulation of aflatoxin biosynthesis. In the *A. parasiticus aflR* transformants, the production of aflatoxin pathway intermediates was significantly enhanced in transformants that contained an additional *aflR* plus *aflS* (Chang et al., 1995). Quantitative polymerase chain reaction (PCR) showed that in the *aflS* knockout mutants, the lack of *aflS* transcript is associated with 5 to 20 fold reduction of expression of some aflatoxin pathway genes such as *aflC* (*pksA*), *aflD* (*nor-1*), *aflM* (*ver-1*) and *aflP* (*omtA*). The mutants lost the ability to synthesize aflatoxin intermediates and no aflatoxins were produced (Meyers et al., 1998).

Other physically unrelated genes, such as *LaeA* and *VeA*, also have been shown to exhibit a "global" regulatory role on aflatoxin biosynthesis. *LaeA* and *VeA* are two critical genes in the formation of velvet complex, a transcriptional complex regulating both sporulation and secondary metabolism in *A. flavus* similar to *A. nidulans* (Amaike and Keller, 2014). *LaeA* only exerts its effect on aflatoxin biosynthesis at a certain level and is independent of other regulatory pathways that are involved in fungal development (Kale et al., 2007). *VeA* deleted *A. flavus* and *A. parasiticus* strains completely lost the ability to produce aflatoxin regardless of the illumination conditions (Duran et al., 2007). Both *LaeA* and *VeA* are required for full virulence. Null mutants of both produce fewer conidia and less aflatoxin in seed and are impaired in lipid degradation of host cells (Amaike and Keller, 2009). Better understanding of the mechanisms of gene regulation on aflatoxin biosynthesis will help to identify natural inhibitors of fungal growth and aflatoxin formation.

In addition to the genetic factors, many biotic and abiotic environmental factors influence aflatoxin biosynthesis including nutritional factors such as carbon and nitrogen source; environmental effects such as water activity and temperature and physiological conditions such as pH and bio-reactive agents (Calvo et al., 2002; Georgianna et al., 2009; Yan et al., 2012). Fungal developmental stage, oxidative stress (Amare and Keller, 2014) and plant metabolites also influence aflatoxin production (Yu, 2012).

1.2.2. Sterigmatocystin

Sterigmatocystin (STERIG) is a secondary fungal metabolite produced by many *Aspergillus* species including *A. flavus*, *A. parasiticus*, *A. versicolor* and *A. nidulans* of which *A. versicolor* is the most common source. Other species such as *Penicillium*, *Bipolaris*, *Chaetomium* and *Emiricella* are also able to produce STERIG (Frisvad et al., 2005; Veršilovskis & De Saeger, 2010; Rank et al., 2011). It is a polyketide-derived furanocoumarin and structurally is closely related to aflatoxins (Fig. 1-6). It is a biogenic precursor of aflatoxin B1 (AFB1). It consists of a xanthone nucleus attached to a

bifuran structure and its most important derivative is O-methyl sterigmatocystin (OMST) (Battilani et al., 2008).

STERIG has been found in grains, nuts, green coffee beans, spices, beer and cheese (EFSA, 2013). It showed different toxicological, mutagenic and carcinogenic effects in animals (EFSA, 2013). The acute toxicity, carcinogenicity and metabolism of STERIG were compared with those of aflatoxins and it has been classified in group 2B as a possible human carcinogen (IARC, 1993a).

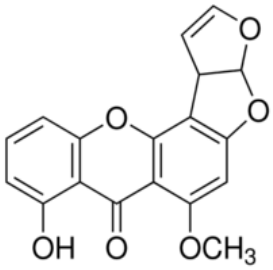


Fig. 1-6. Structure of sterigmatocystin

1.2.3. Ochratoxins

Ochratoxins are a group of pentaketide metabolites, comprised of a dihydroisocoumarin bonded to phenylalanine (Pohland et al., 1992). The most common congener ochratoxin A (OTA) (Fig. 1-7) is mainly produced by *Aspergillus ochraceus. Penicillium verrucosum* and other *Aspergillus* species are known to produce ochratoxins A, B and C (Bennet an Klich, 2003). Ochratoxins are common contaminants in grains, coffee, cocoa, spices, wine, beer and pork (Bayman and Baker, 2006). It is a potent nephrotoxin, classified as a group 2B possible human carcinogen (IARC, 1993a). It has been suggested as a factor in the etiology of a human disease known as Balkan endemic nephropathy (BEN). Moreover, it has been shown to be hepatotoxic, teratogenic and immunotoxic (O'Brien and Dietrich, 2005).

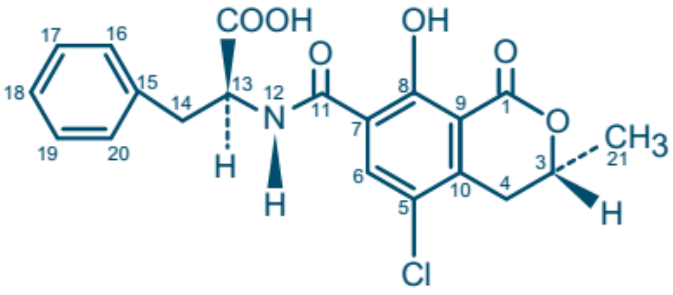
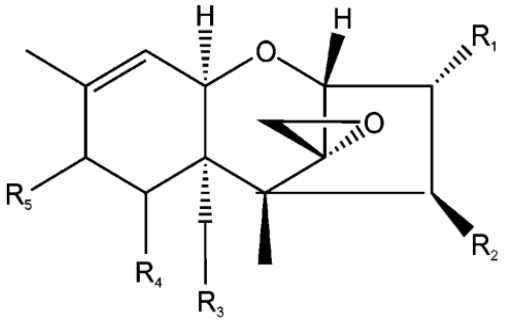


Fig. 1-7. Chemical structure of ochratoxin A

1.2.4. Trichothecenes

Approximately 170 identified trichothecenes are classified in four types (A-D) based on the differences in functional hydroxyl and acetoxy side groups (Groove, 1988 & 1993). All the trichothecenes are characterized by a tetracyclic 12,13-epoxy trichothecene skeleton (Fig. 1-8). Several naturally occurring trichothecene mycotoxins are produced in foods and feeds by *Fusarium* species, e.g., Type A: T-2 toxin, HT-2 toxin, diacetoxyscirpenol (DAS) and Type B: deoxynivalenol (DON; vomitoxin), nivalenol (NIV), Fusarenon X (FUS-X), 3-acetyldeoxynivalenol (3-ADON) and 15-acetyldeoxynivalenol (15-ADON) (CAST, 2003). Trichothecenes are known for

their strong capacity to inhibit eukaryotic protein biosynthesis. DON, the most common trichothecene found in grains, is non-classifiable as carcinogenic to human (IARC, 1993b), but it can cause deleterious health effects like anorexia, malnutrition, weight loss, endocrine dysfunction and immune alterations (Pestka, 2010).



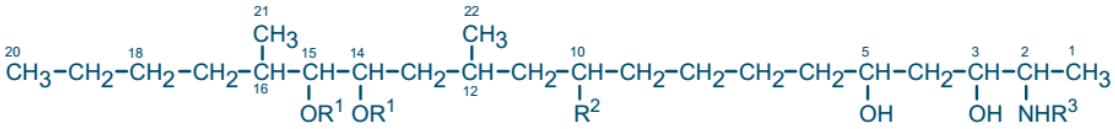
	Trichothecene	MW	R_1	R_2	R_3	R_4	R_5
	T-2 toxin	466.5	OH	OAc	OAc	H	OCOi-Bu
	HT-2 toxin	424.5	OH	OH	OAc	H	OCOi-Bu
Type A	15-Monoacetoxyscirpenol	324.4	OH	OH	OAc	H	H
	Diacetoxyscirpenol	366.4	OH	OAc	OAc	H	H
	Neosolaniol	382.4	OH	OAc	OAc	H	OH
	Deoxynivalenol	296.3	OH	H	OH	OH	=O
	Nivalenol	312.1	OH	OH	OH	OH	=O
Type B	3-Acetyldeoxynivalenol	338.4	OAc	H	OH	OH	=O
	15-Acetyldeoxynivalenol	338.4	OH	H	OAc	OH	=O
	Fusarenon-X	354.4	OH	OAc	OH	OH	=O

Fig. 1-8. Structure of Type A and Type B trichothecenes

1.2.5. Fumonisins

Fumonisins consist of a group of substances composed of a carbon-chain back bone with two tricarballylic acid groups esterified at the C14 and C15 positions (CAST, 2003) having four carboxylic acid groups (Fig. 1-9). Many fumonisins are discovered recently. These include the FBX series (12 compounds) with the tricarballylic acid moiety replaced by other carboxylic acids, the FD series with fewer carbon atoms in the backbone and numerous other related metabolites. Twenty eight isomers of FB1 were detected by LC-MS/MS in *F. verticillioides* cultures (Bartók et al., 2010; Scott, 2012). Fumonisins are mainly produced by *Fusarium verticillioides* (syn., *moniliforme*) and *Fusarium proliferatum* (Gelderblom et al., 1988). *Fusarium napiforme, Fusarium dlamini* and *Fusarium nygamai* are also able to produce fumonisins (EFSA, 2005). They can frequently contaminate maize and cereal grains, growing best at high temperature and humidity conditions (Marín et al., 1995), thus, they can co-occur with aflatoxins in corn (Kimanya et al., 2008; Sun et al., 2011).

It is nephrotoxic and hepatotoxic and classified as group 2B, possible human carcinogen (IARC, 2002; JECFA, 2012). It has been associated with liver and oesophageal cancers in high risk populations (Chu and Li, 1994; Persson et al., 2012).



Fumonisins

A_1: $R^1 = COCH_2CH(CO_2H)CH_2CO_2H$; $R^2 = OH$; $R^3 = OH$; $R^4 = C^{23}OC^{24}H_3$
A_2: $R^1 = COCH_2CH(CO_2H)CH_2CO_2H$; $R^2 = H$; $R^3 = OH$; $R^4 = COCH_3$
B_1: $R^1 = COCH_2CH(CO_2H)CH_2CO_2H$; $R^2 = OH$; $R^3 = OH$; $R^4 = H$
B_2: $R^1 = COCH_2CH(CO_2H)CH_2CO_2H$; $R^2 = H$; $R^3 = OH$; $R^4 = H$
B_3: $R^1 = COCH_2CH(CO_2H)CH_2CO_2H$; $R^2 = OH$; $R^3 = H$; $R^4 = H$

Fig. 1-9. Structure of fumonisins

1.2.6. Zearalenone

Zearalenone (ZEN), an estrogenic mycotoxin (Fig. 1-10), produced primarily by *Fusarium graminearum* and *Fusarium culmorum*, occurs in wheat, barley, sorghum and high-moisture corn and has also been found in moldy hay and pelleted feeds (CAST, 2003). ZEN producing fungi are mainly found in temperate conditions and high contamination in cereals are associated with wet temperate weather and improper storage in high moisture environments (Marroquin-Cardona et al., 2014). It is not classifiable as a human carcinogen (IARC, 1993b), however it is potent due to its estrogenic activity. It highly resembles 17-β-estradiol, the principal hormone produced in human ovaries thus binding to the estrogen receptors in mammalian target cells (Bennet and Klich, 2003). As such it can cause precocious puberty in girls (Massart et al., 2008).

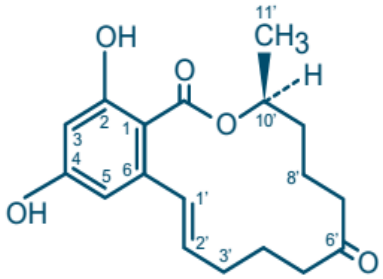


Fig. 1-10. Structure of zearalenone

1.2.7. Citrinin

Citrinin is a yellow polyketide mycotoxin (Fig. 1-11) first isolated from *Penicillium citrinum*, and later determined to be produced by several *Penicillium* and *Aspergillus* species, including strains of *P. verrucosum* that also produce ochratoxin. It has structural similarity with OTA and like OTA, citrinin causes kidney damage in laboratory animals similar to swine nephropathy. Citrinin is heat sensitive and decomposes during heat treatment to form other complex compounds, such as citrinin H1 and citrinin H2, respectively with higher and weaker cytotoxicity than the original citrinin (EFSA, 2012). Citrinin can be found in rye, barley, corn, maize and wheat grains (Bennet and Klich, 2003). Citrinin is known to occur also as an undesirable contaminant in Monascus fermentation products (red mould rice (RMR)), which have been used in Asia for centuries for meat preservation and food colouring (EFSA, 2012).

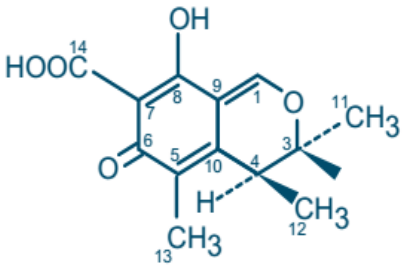


Fig. 1-11. Structure of citrinin

1.2.8. Alternaria toxins

Alternaria toxins are mycotoxins produced by *Alternaria* species that cause plant diseases on many crops in the field or during the post-harvest period. They are the principal contaminating fungi in wheat, sorghum and barley, and have also been reported to occur in oilseeds such as sunflower and rapeseed, tomato, apples, citrus fruits, olives and several other fruits and vegetables (EFSA, 2011a; Van de Perre, 2014). The most abundant fungi of the forty *Alternaria* species, *A. alternata* produces alternariol (AOH), alternariol methyl ether (AME), altenuene (ALT), altertoxins I, II, III, tenuazonic acid (TeA) (Fig. 1-12) and other less toxic substances (Ostry, 2008). These toxins show cytotoxic, fetotoxic and/or teratogenic activity, they are mutagenic, clastogenic and oestrogenic in microbial and mammalian cell systems. They are also reported to be tumorigenic in rats and to inhibit the cell proliferation (Müller and Korn, 2013).

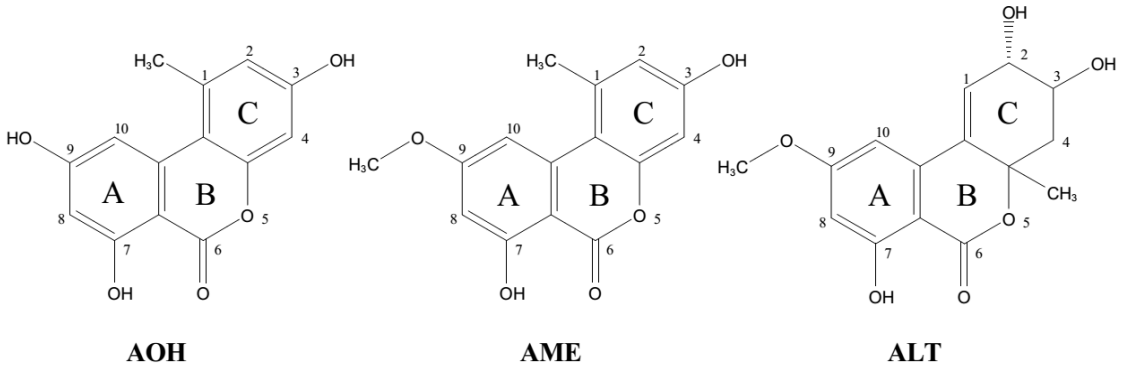


Fig. 1-12. Structures of alternaria toxins: alternariol (AOH), alternariol methyl ether (AME), altenuene (ALT)

1.3. ANALYTICAL TECHNIQUES FOR THE DETERMINATION OF MYCOTOXINS

The fact that most mycotoxins are toxic at very low concentrations requires sensitive and reliable methods for their detection. The application of simpler, cheaper and effective techniques for the detection of mycotoxins is increasingly being required due to their perceived importance based on their toxicity and legislation requirements (Turner et al., 2009).

Determination of mycotoxins involves sample pre-treatment, separation and detection methods. Sample pre-treatment or a clean-up is vital in successful mycotoxin analysis, depending on the compound properties and complexity of the matrix. Solid phase extraction (SPE) is the most popular technique used in routine mycotoxin analysis however, liquid-liquid extraction and super-critical fluid extraction have also been used. Several chromatographic methods are available for separation and detection of multi-class mycotoxins. Traditionally, the most popular method was thin layer chromatography (TLC). Use of TLC analysis is still common in developing countries because of its high throughput and low cost (Krska et al., 2008; Lin et al., 1998).

Modern analysis heavily depends on high performance liquid chromatography (HPLC) using various adsorbents depending on the structure of mycotoxins. Quantitative methods for aflatoxins, OTA, fumonisins, ZEN and trichothecenes T-2 toxin and DON in food and feed using immuno-affinity cleanup with HPLC or gas chromatography (GC) in combination with various detectors, like fluorescence detector (FLD) (with either pre- or post-colum derivatisation), ultra-violet (UV) or flame ionisation detection (FID), electron capture detection (ECD) and mass spectrometry (MS) are available (Krska and Molinelli, 2007).

Liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) stands out in mycotoxin analysis methods these years because of its universal, selective and sensitive detection. Increasing interest toward multiple mycotoxins analysis has so far been best achieved by LC-MS/MS, enabling simultaneous determination of a wide range of multi-class mycotoxins in a single run. Reduction or possible omission of sample pre-treatment became possible due to the highly sophisticated LC-MS interfaces. Hundreds of U(H)PLC-MS/MS based multi-mycotoxins and multi-substrate methods have emerged over the past decade in food (Sulyok et al., 2007; Spanjer et al., 2008; Shepard, 2011; Kadota et al., 2011; Hajslova et al., 2011; Monbaliu et al., 2010; De Boevre et al., 2012; Njumbe Ediage et al., 2011; Krska et al., 2012; Yogendrarajah et al., 2013), feed (Rasmussen et al., 2010; Di Mavungu et al., 2012) and biological fluids (Solfrizzo et al., 2011; Turner et al., 2012; Njumbe Ediage et al., 2012; Warth et al., 2012a). Moreover, LC-high resolution mass spectrometry (HRMS) is currently being applied in multi-mycotoxins analysis (Lattanzio et al., 2011; Zachariasova et al., 2010), in masked or modified forms and for the identification of new secondary fungal metabolites (Latanzio et al., 2012; Sanchez et al., 2012; Zachariasova et al., 2012; Malysheva et al., 2014).

Analytical developments in mycotoxin analysis have been continuously reviewed over the years (Shepard et al., 2009; 2010; 2011; 2012). Trends in the application of fast LC techniques, including sample preparation and chromatographic approaches were recently summarised by Núñez et al. (2012). Some general principles of sample clean-up with developments in sol-gel immuno-affinity chromatography, aptamers and molecularly imprinted polymers (MIPs) have also been reviewed (Cichna-Markl, 2011), whereas others have reviewed the application of hyphenated techniques for characterization and determination of masked mycotoxins (Cirlini et al., 2012). The application of biosensors, the potential use of nano-materials in the development of rapid methods (Tothill, 2011; Maragos and Bunsen, 2010), the use of lateral flow devices and surface plasmon resonance (SPR)-based sensors for detecting mycotoxins (Anfossi et al., 2012; Li et al., 2012) have been reviewed as well.

1.4. RISK ASSESSMENT OF MYCOTOXINS

1.4.1. General introduction

The mycotoxin problem in public health is long standing and all humans and animals are at risk for mycotoxins exposure at various extents. The worldwide occurrence of mycotoxins in foods and feeds has been recognized by the FAO and World Health Organization (WHO) for many years (FAO, 2001). It is highly influenced by certain environmental factors, hence the extent of contamination of a particular commodity with a particular toxin is unpredictable and will also vary with geographical location, agricultural practices, and the susceptibility of commodities to fungal invasion during pre-harvest, storage and/or processing periods (CAST, 2003) (Fig. 1-13).

Approximately, 5-10% of agricultural products worldwide are spoiled by fungi, to the extent that crops cannot be consumed by humans or animals. Furthermore, the FAO estimates that more than 25% of the agricultural produce is contaminated by mycotoxins (FAO, 2004).

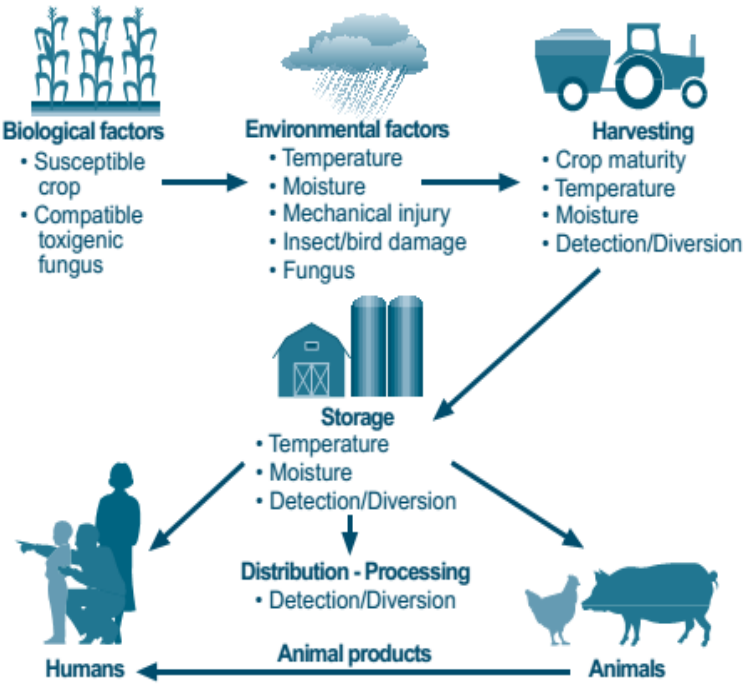


Fig. 1-13. Factors affecting mycotoxin occurrence in the food and feed chain (CAST, 2003)

As described in the previous section, many mycotoxins exert potent acute and chronic biological activities determined by animal studies, in vitro-bio assays and human epidemiological studies. Comprehensive reviews on the human health effects caused by mycotoxins are available (JECFA, 2001b; IARC, 2012; Richard, 2007). In terms of exposure and severity of chronic disease, especially cancer, mycotoxins appear to pose a higher risk than anthropogenic contaminants, pesticides and food additives (Table 1-1). Thus, from a public health point of view there is high concern regarding the health effects associated with long-term exposure to low levels of mycotoxins.

Acute toxicity of mycotoxins is rarely observed in developed countries (CAST, 2003). However, in many low-income countries where agricultural practices and regulations are least adapted, mycotoxins affect staple foods, groundnuts, maize and other cereal grains. Hence exposure is continuous and often at high levels (Wild and Gong, 2010). The reason for the lack of action to

tackle the problem of mycotoxins in low-income countries has become unquestionably complex and incompletely researched (Wild, 2007). To achieve practical solutions that balance the need to protect health with economic concerns, risk analysis could serve as an indispensable tool.

Table 1-1. Rating of health risks from foods

Acute	Toxicity	Chronic
Microbiological	High	**Mycotoxins**
Phycotoxins		Anthropogenic contaminants
Some phytotoxins		Some phytotoxins
Mycotoxins		Unbalanced diet
Anthropogenic contaminants		Phycotoxins
Pesticide residues		Food additives
Food additives		Pesticide residues
	Low	Microbiological

Source: Kuiper-Goodman, 1998

In general, risk analysis is an iterative process that systematically and transparently collects, analyses and evaluates relevant information about chemical, biological or physical hazards possibly associated with food. As a structured decision making process it constitutes three distinct and closely connected components, risk assessment, risk management and risk communication (FAO/WHO, 2006) (Fig. 1-14).

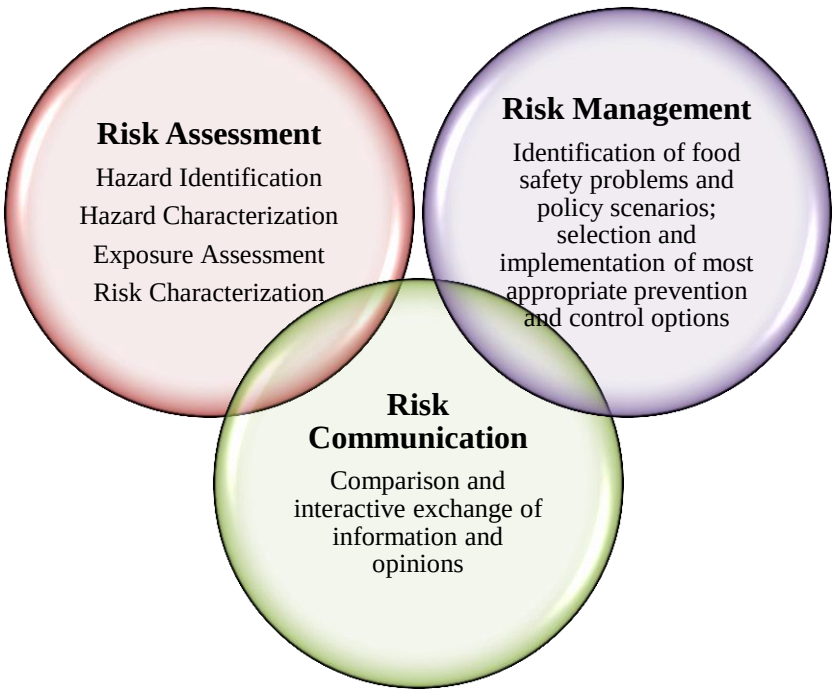


Fig. 1-14. Risk analysis framework (Based on Benford, 2001)

Risk assessment is the scientific evaluation of the probability of the occurrence of known or potential adverse health effects resulting from human exposure to food-borne hazards. It is the primary scientific basis for promulgation of regulations which aim at managing the risk. The first two risk assessment components, hazard identification and hazard characterization are related to the universal properties of the contaminant.

1.4.2. Hazard identification

Hazard identification has been defined as ‘‘the identification of biological, chemical and physical agents capable of causing adverse health effects and which may be present in a particular food or group of foods’’ (FAO/WHO, 2006). Many mycotoxins and toxigenic fungi were discovered because they were causally associated with mycotoxicoses affecting humans (ergotism, liver cancer, yellow rice disease, alimentary toxic aleukia (ATA), BEN, red mould toxicosis) and animals (turkey-X-disease by aflatoxins, porcine nephropathy by OTA, vulvovaginitis in pigs by ZEN, equine leuko encephalomalacia and porcine pulmonary edema by fumonisins) (Richard, 2007; Kuiper-Goodman, 2004).

1.4.3. Hazard characterization

Hazard characterization has been defined as ‘‘the qualitative and/or quantitative evaluation of the nature of the adverse health effects associated with biological, chemical and physical agents, which may be present in food” (FAO/WHO, 2006). A hazard is characterized by establishing a dose-response relationship between different levels of exposure to the hazard in food and at the point of consumption and the likelihood of different adverse health effects usually due to long term exposure. However, for hazards like mycotoxins both chronic and acute effects need to be considered.

1.4.3.1. Non-carcinogenic mycotoxins: Since “only the dose makes the poison”, for cases where non-carcinogenic adverse effects were observed in animals and humans, usually a health based guidance value (HBGV) or tolerable daily intake (TDI) could be established (FAO/WHO, 2006). The TDI is the dose that can be consumed daily over a life time without incurring appreciable adverse health effects, which by implication therefore involves a biologically insignificant risk (WHO, 1999; Edler et al., 2002). The TDI is generally derived *via* the determination of a no observed adverse effect level (NOAEL), below which adverse effects are not observed. The TDI is derived by dividing the NOAEL obtained from animal studies by a safety (uncertainty) factor of 100 when extrapolating to humans, which considers a 10 fold factor for inter-species variation and another 10-fold for intra-species variability (Edler et al., 2002). This approach was used by JECFA to assign provisional maximum tolerable daily intakes (PMTDI) for fumonisins, OTA, DON and T-2 toxin (JECFA, 2001b & 2007). TDIs (PTDI (provisional TDI) or

PTWI (provisional tolerable weekly intake)) set by various organizations for the more frequently occurring non-genotoxic mycotoxins occurring in food are shown in Table 1-2.

Table 1-2. Health based guidance values (HBGV) for the most frequently occurring non-genotoxic mycotoxins in food

Mycotoxin	HBGV	References
OTA	PTWI=100 (ng/kg bw[b]/week)	JECFA, 2007
	PTWI=120 (ng/kg bw/week)	EFSA, 2006
Fumonisins[a]	TDI=2 (µg/kg bw/day)	SCF, 2003; JECFA, 2001
Sum of T-2 and HT-2	TDI=100 (ng/kg bw/day)	EFSA, 2011b
Patulin	PTDI=0.4 (µg/kg bw/day)	SCF, 2000a
ZEN	TDI=0.25 (µg/kg bw/day)	EFSA, 2011c
Nivalenol	PTDI=0.7 (µg/kg bw/day)	SCF, 2000b
DON and ADONs	PMTDI=1 (µg/kg bw/day)	SCF, 2002

[a]FB1, FB2 and FB3, alone or in combination; [b]body weight.

1.4.3.2. Carcinogenic mycotoxins: With regard to carcinogenic myctoxins, it is generally presumed that there is no threshold dose below which there is no induction of cancer initiation, thus there would always be some risk even at lower doses, unless it was clearly established that the mode of action involves an indirect mechanism that may have a threshold (Kuiper-Goodman, 2004). AFB1 is a DNA-reactive carcinogenic mycotoxin that appears to have both initiating and promoting properties and that may also contribute to tumor progression. Thus, a TDI is generally not determined. Several other carcinogenic mycotoxins appear to be non-DNA reactive or are indirectly DNA-reactive.

1.4.4. Exposure assessment

Exposure assessment has been defined as ‘‘the qualitative and/or quantitative evaluation of the likely intake of biological, chemical or physical agents *via* food, as well as exposures from other sources if relevant’’ (FAO/WHO, 2006). Whereas hazard identification and characterization relate to universal properties of the contaminant, exposure assessment is variable across populations and subgroups of populations. It depends on the level of the substances that are present in different foods and on the intake of these foods (Kroes et al., 2002). Reliable and validated analytical tools are available now for many mycotoxins and also for different food commodities. Mycotoxins in food occur frequently at low concentrations, with a number of samples having concentrations below the limit of detection (LOD) or limit of quantification (LOQ). Thus, treatment of the “non-detects” can have an important influence in exposure estimations (Kuiper-Goodman, 2004; EFSA, 2010). There can be large national and international regional differences in food intakes thus exposure

assessments are country or even regional specific. Where national dietary surveys are available, these consumption data can be combined with the contamination data to estimate the exposure. Traditionally, exposure estimates have been point estimates (deterministic exposure assessment), where fixed values of mean or percentiles of both contamination and consumption data were used (FAO/WHO, 2006). The introduction of probabilistic exposure assessment in recent years allows to examine the distribution of exposure from a convolution of all the underlying distributions using Monte Carlo based simulations.

Exposure estimations of mycotoxins can also be based on the measurements of serum or urinary biomarkers in human (Njumbe Ediage et al., 2013; Warth et al., 2012b) and can then be assessed based on pharmacokinetic relationships (Kuiper-Goodman, 2004). The advantage of this approach is that in addition to considering different pathways it also takes into account the bioavailability from food matrices (O'Brien et al., 2006). Biomarkers are available for aflatoxins, OTA, fumonisins and DON (Crews et al., 2001; Meky et al., 2003; Coronel et al., 2010; Shepard et al., 2007; Turner et al., 2009; Turner et al., 2012).

1.4.5. Risk characterization

Risk characterization has been defined as ''the qualitative and/or quantitative estimation, including attendant uncertainties, of the probability of occurrence and severity of known or potential adverse health effects in a given population based on hazard identification, hazard characterization and exposure assessment'' (FAO/WHO, 2006). For chemical hazards, different approaches have been adopted for the risk characterization of those with a threshold and without a threshold (Renwick et al., 2003). The output of the exposure assessment of the mycotoxins with a threshold is finally compared with the TDI in order to determine whether the estimated exposures are within or above the safe limits. For the chemical without a threshold following four different approaches can be used (Barlow et al., 2006),

1) As low as reasonably achievable (ALARA)
2) Low-dose extrapolation of data from rodent carcinogenicity bioassays
3) Threshold of toxicological concern (TTC)
4) Margin of exposure (MoE)

Although the ALARA principle is an easy concept, it does not discriminate between very potent and very weak carcinogens and it does not take into account the human exposure (FAO/WHO, 2006). Moreover, a simple linear extrapolation approach might overestimate the real risks, thus it is not recommended (O'Brien et al., 2006). Threshold of Toxicological Concern (TTC) is a concept that aims to establish a level of exposure for all structurally related chemicals below which there would be no appreciable risk to human health. TTC is the daily intake associated to give a life time risk of

less than 1 in a million. A generic threshold of 0.0025 µg/kg BW/day is applied for structural alerts (a particular chemical grouping within a chemical structure which is known to be associated with a particular type of toxic effect (Barlow, 2005)) and used only when there is no good hazard characterization (Kroes et al., 2004). The Cramer classification scheme (tree) is probably the best known approach for structuring chemicals in order to make a TTC estimation. The tree relies primarily on chemical structures and estimates of total human intake to establish priorities for testing (Cramer et al., 1978).

The margin of exposure approach has been applied to a number of contaminants including aflatoxins (EFSA, 2005b). The MoE is defined as the ratio of the NOAEL or benchmark dose lower confidence limit (BMDL) for the critical effect to the theoretical, predicted or estimated exposure dose or concentration (WHO, 2009). MoE has been accepted by JECFA as the best approach to characterize the risk of substances that are genotoxic and carcinogenic (FAO/WHO, 2006). EFSA concluded that the magnitude of an MoE could be used by risk managers for priority setting and was more informative than advising that exposures should be reduced to ALARA (EFSA, 2005b).

Since it is considered scientifically invalid to identify a NOAEL for substances that are genotoxic and carcinogenic MoE is calculated from a point of departure (PoD) (a reference point) on the dose-response relationship from experimental or epidemiological studies. The benchmark dose (BMD) approach offers the best tool for deriving a suitable PoD in the observable dose–response range. The dose that causes a low but measurable response (benchmark response (BMR) of typically 5% or 10%) is designated as the BMD, and its lower 95% confidence limit is the BMDL (Fig. 1-15). MoE is considered to be the most scientifically credible and practical approach for the formulation of advice because it takes into account both the dietary exposure and the available data on the dose–response relationship, i.e. potency, without extrapolation beyond the observed dose-range or generation of uncertain risk estimates (Barlow et al., 2006).

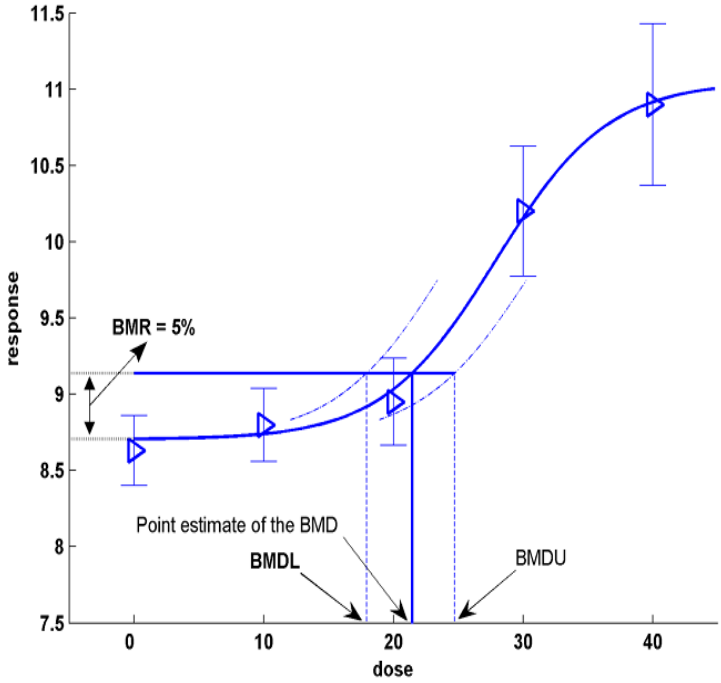


Fig. 1-15. Key concepts of the BMD approach (EFSA, 2009).

The magnitude of the MoE gives an indication of the level of concern, but is not a precise quantification of risk. The larger the MoE, the smaller the potential risk posed by exposure to the compound under consideration. EFSA considered that an MoE of 10,000 or more, based on animal cancer bioassay data, ‘‘would be of low concern from a public health point of view and might reasonably be considered as a low priority for risk management actions” (EFSA, 2005b; Barlow and Schlatter, 2010).

Quantitative cancer risk assessment models have been applied to mycotoxins that are genotoxic carcinogens (FAO/WHO, 2006). These models employ biologically-appropriate mathematical extrapolations from high-dose animal cancer incidence data to calculate the cancer incidence associated with ordinary human exposure. Since AFB1 exposure has been correlated with human liver cancer, two separate cancer potencies have been suggested based on the interaction between hepatitis B and aflatoxin exposure; 0.3 for populations in which chronic hepatitis infections are common and 0.01 for rare infection cases (JECFA, 1998).

1.4.6. Risk management of mycotoxins

Mycotoxins are unavoidable contaminants, thus risk management is necessary to ensure that exposure to mycotoxins does not pose a health risk. Priorities for risk management depend on the extent and frequency that the TDI is exceeded or on the size of the MoE (Health Canada, 1994). Thus, risk assessment has become the basis of establishing regulations. With regulations in place the exposure to mycotoxins should normally not exceed the TDI. Regulations are generally effective in controlling mycotoxins in industrialized nations, while in less developed countries (LDC) they do little to protect public health, as there is limited enforcement of food safety regulations (Wu and Khlangwiset, 2010a) and food scarcity makes the issue even more complicated.

In addition to these regulations, many other interventions have been developed either to reduce mycotoxins directly in the field and in food (pre- and post-harvest interventions) or to reduce their harmful effects in the body once it is ingested (dietary and clinical interventions) (Turner et al., 2005; Khlangwiset and Wu, 2009; Wu and Khlangwiset, 2010a & 2010b; Wild and Hall, 2000; Wu, 2014). The emerging risk management metrics, Food Safety Objectives (FSO), Appropriate Level of Protection (ALOP), Performance Objectives (PO) and Performance Criteria (PC) could also be applied in mycotoxins management (Garcia-Cela et al., 2012).

1.4.7. Risk communication

Risk communication has been defined as an interactive exchange of information and opinion throughout the risk analysis process concerning risk, risk related factors and risk perceptions among risk assessors, risk managers, consumers, industry, the academic community and other interested parties, including the explanation of risk assessment findings and the basis of risk management decisions (CAC, 2003). EFSA contributes to improving food safety in Europe and to build public confidence in the way risk is assessed by communicating on risks in an open and transparent way based on the independent scientific advice of its scientific expert panels (EFSA, 2010). It aims to deliver the best science, at the right time and in the most appropriate manner, through effective cooperation in risk communications with all key actors. According to EFSA (2010), the risk communications strategy sets out the following key priorities:

- Simplicity and transparency - increase relevance and understanding of EFSA communications for key target audiences and informed lay audiences, in co-operation with Member States.
- Independence - augment proactive communications on the independence of EFSA's risk assessment advice.
- Visibility and outreach - enhance outreach, in the EU and beyond, by increasing awareness and recognition of EFSA and its role and work as risk assessor.
- Coherence - further increase the coherence of risk communications across the EU and beyond.
- Dialogue - enhance dialogue with stakeholders and increase audience interactivity.

According to FAO/WHO (2005), risk communication is a powerful but neglected tool in helping people to make informed choices about risks. For any communication about risk caused by a specific event to be effective, it needs to taken into account the social, religious, cultural, political and economic aspects associated with the event, as well as the voice of the affected population. Communications of this kind promote the establishment of appropriate prevention and control action through community-based interventions at individual, family and community levels.

1.5. REGULATION OF MYCOTOXINS

Regulations relating to mycotoxins have been established in many countries to protect the consumer from the harmful effects of these compounds. As it is impossible to fully eliminate food contamination, maximum levels (MLs) should be set at a strict level which is reasonably achievable by following good agricultural and manufacturing practices and taking into account the risk related to food consumption. Thus, risk assessment is the main scientific basis for the setting of mycotoxin regulatory limits (Wu, 2004). Distribution of the concentration of mycotoxins in products is an important factor to be considered in establishing regulatory sampling criteria. As mentioned earlier, reliable analytical methods will have to be available to make enforcement of the regulations possible. Apart from the scientific factors, economic factors such as commercial and trade interests and food security issues also have an impact. Despite these difficulties, mycotoxin regulations have been established in at least 100 countries (covering approximately 85% of the world's inhabitants) and for 13 different (groups of) mycotoxins during the past decades, and newer regulations are still being issued (Van Egmond et al., 2007). Regulations relating to mycotoxins on a global and European context were lastly reviewed in detail was in 2007 (Van Egmond et al., 2007).

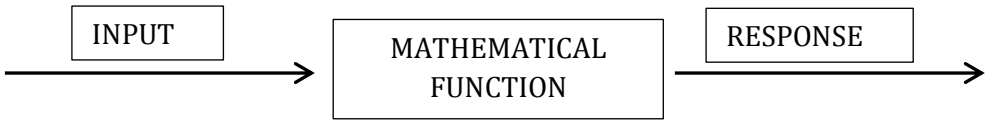
International legislation on foods and feeds is established by Codex Alimentarius Commission (CAC). The Codex Alimentarius system for development of legislation concerning contaminants, including mycotoxins in foods and feeds, is laid down (CAC, 2000). Since the emergence of large economic communities mycotoxin regulations are being harmonized more and more between countries belonging to the same economic communities (e.g. EU, 'Mercado Cómun del Sur' or Southern Common Market (MERCOSUR), Australia/New Zealand, Association of Southeast Asian Nations **(**ASEAN), overruling existing national regulations. The EU has implemented the most comprehensive regulations for food mycotoxins worldwide (Van Egmond et al., 2007). Current regulations are increasingly based on scientific opinions of authoritative bodies, the JECFA and EFSA. The EC has established ML for certain mycotoxins in some foodstuffs which is originally published in Commission Regulation (EC) No 1881/2006 (EC, 2006b). Commission Regulation (EC) No 1126/2007 sets MLs for *Fusarium* toxins in maize and maize products (EC, 2007). Aflatoxins and OTA in spices (*Capsicum* spp., *Piper* spp., nutmeg, ginger, turmeric and mixture containing one or more of these spices) are controlled by the EU regulations 165/2010 (EC, 2010a) and 105/2010 (EC, 2010b), respectively. Current limits for the levels of aflatoxins in spices worldwide are described by Zhao et al. (2013). The most common limit for AFB1 in spices is 5 µg/kg, while 10 µg/kg is the most common limit for the combined AFB1, B2, G1 and G2. A ML of 30 µg/kg is considered for combined aflatoxins for all foods in Sri Lanka.

1.6. FUNGAL PREDICTIVE MODELLING/"PREDICTIVE MYCOLOGY"

1.6.1. General introduction

Predictive microbiology can be considered as a scientific branch of the food microbiology field intended to quantitatively assess the microbial behavior in food environments to derive adequate mathematical models (Pérez-Rodríguez and Valero, 2013). A mathematical model is a description of a real system by using mathematical equations, which are simplifications of the system based on its more significant properties.

A basic model is structured as,



The mathematical model estimates the response of the represented system or process based on the values of the input variables. The need to assess the risk from pathogenic organisms in food products has led to the development of mathematical models to quantify and predict microbial behavior (Lahlali et al., 2005; McMeeekin et al., 2008). Although the first predictive models were dated at the beginning of the 20th century, its great development has occurred in the past decade as a result of computer software advances. A large number of studies on predictive modelling are available in literature however most of them were focusing on food-pathogenic and spoilage bacteria.

Predictive modelling of filamentous fungal growth has not received the same level of attention as that of bacterial growth. This may be because of the inherent complexities associated with the quantification of fungal growth. Fungal development involves germination followed by hyphal extension. Visible mycelium appear shortly after germination is completed. Germination requires microscopic observation for evaluating the length of the germ tube (Dantigny et al., 2005a). Whereas, mycelial growth usually reported as radial growth rate (mm/h or mm/day), is mostly determined by evaluating temporal colony diameter changes macroscopically. It is probably the simplest and most direct measure, but it may not necessarily represent the true nature of fungal growth. Whereas bacteria reproduce by fission and growth normally takes place only at surfaces or homogeneously through a liquid or visco-elastic medium, after germination fungal hyphae can penetrate the physical three-dimensional matrix of foods (Gibson and Hocking, 2007; Dantigny et al., 2003 and 2005a). Hence, for a long time there were no simple direct methods to estimate fungal growth with respect to time. A pre-requisite for producing a useful and reliable model must be a database containing large amounts of relevant data, preferably in the form of growth or survivor curves. Obtaining reproducible and similar quality growth curves for fungi as of bacteria remains much more complicated (Gibson and Hocking, 2007).

Due to the appearance of visible hyphae and production of unpleasant odours, fungal spoilage of food causes economic losses. It has been very difficult to assess losses attributable to moulds. As stated previously, 25% of the world's agricultural produce are contaminated with mycotoxins (FAO, 2004). Hence, there is a need to develop a tool for the prediction of fungal development and mycotoxin production. The tendency to extend the use of models that were developed for bacteria to moulds has been first proposed by Dantigny et al. (2003 and 2005a). However, bacterial growth models cannot be translated always directly to predict the growth behaviour of moulds. According to Dantigny et al. 2003 and 2005a, in modelling fungal kinetics, the tools that were developed for bacteria can be used, but mould specificities should be taken into account.

Hence, the concept of "Predictive Mycology" was introduced, which can serve as a useful tool for the determination of mould or mycotoxin contamination in food. Since then several studies were dedicated on predicting different fungal species. However, predictive mycology still borrows lot of techniques from the main stream predictive microbiology (Gibson et al., 1994; Dantigny et al., 2005a). Colony diameters have been mostly used to estimate the fungal growth rate since they better correlate with fungal growth than CFU counts (Marín et al., 2005). Few scientific reviews on fungal modelling have been published. The first review was published by Gibson and Hocking (1997). Years later, Dantigny et al. (2005a), developed the basis of predictive mycology as explained before, by identifying primary and secondary models applied to germination and mycelium proliferation of spoilage moulds. Additionally, Pardo et al. (2006) and Garcia et al. (2009) reviewed fungal growth and mycotoxin modelling with more focus on food safety.

Predictive microbial models can be broadly classified into two main categories according to the objectives of their use: Kinetic and Probabilistic models. Generally applied models in predictive mycology are described below.

1.6.2. Kinetic predictive models applied to filamentous mould growth

These models are used to determine the mould response in relation to time and environmental conditions, and provide parameter estimations of growth. Predictive models describing kinetic processes are classified as primary, secondary and tertiary models.

1.6.2.1. Primary models

Primary models describe how the number of microorganisms in a population changes with time under specific conditions (Marks et al., 2007), thus accounting for the concentration changes versus time. Primary microbial models can be classified as inactivation, germination and growth models.

1.6.2.1.1. Inactivation models

Inactivation can be described by the Bigelow model. The inactivation of the spores was modelled using a first order classical equation dN/dt = -*k*N. The decimal reduction time or D value (min) is a very important parameter describing the time needed to inactivate 90% of the spores at a given temperature, D=ln(10)/k, where k is the inactivation rate (min^{-1}). The Z value (°C) is another important tool used to determine the temperature increase required to have a 10-fold increase of the D value (Dantigny et al., 2005).

1.6.2.1.2. Germination models

Germination is considered to be very important from the food quality point of view because mould spoilage is observed as soon as visible hyphae are formed in the product. To assess fungal development, primary models can be used to mathematically describe germination and mycelium proliferation (Dagnas and Membré, 2013; Dantigny et al., 2007). Marín et al. (1996), modelled the germination of spores of *Fusarium moniliforme* as a function of time using the Gompertz equation (Eq. 1-1).

$$P = P_{max} \exp\left\{-\exp\left[\left(\frac{\mu_m \exp(1)}{P_{max}}\right)(\delta - t) + 1\right]\right\} \qquad \text{Eq.1-1}$$

where P is the percentage germinated spores (%), P_{max} is the maximal percentage of the germinated spores (%), μ_m is the Gompertz rate (slope term at the point of inflexion for the rate of increasing germinated spores) (h^{-1}) and δ is the lag phase prior germination (h). An asymmetric model can also be used in estimating germination (Dagnas and Membré, 2013). A logistic model has been recently applied for germination time of *Alternaria* spp. in tomato (Van de Perre, 2014).

1.6.2.1.3. Growth models

Mycelium formation and the growth of hyphae can be followed macroscopically. A mould growth curve can be obtained by following the colony diameter increase over time. Mould growth curve follows a pattern consisting of a lag and a linear phase under optimal conditions (or exponential growth in area) (Fig. 1-15). Under sub-optimal conditions, a stationary phase may appear during which fungi stop growing (e.g., under limiting conditions of a_w) (Garcia et al., 2009). Lag time is the period required for fungi to adjust to the new environment and depends on the physiology of the cells and the environmental conditions. The linear phase is usually characterized by remarkable reproducibility. It continues until a 'maximum population density' is reached and then slows down and eventually stops. This is the stationary phase, which is independent of the environmental factors and food is already too spoiled, thus little attention is paid in modelling it (Ross and McMeekin, 2003).

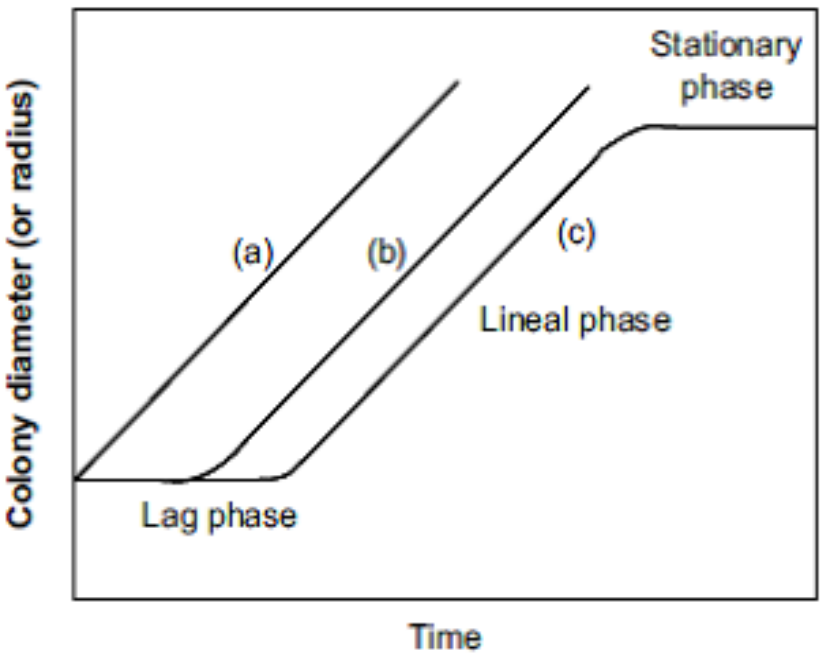


Fig. 1-15. Usual growth curves for moulds under optimal and suboptimal conditions (a) Lineal model, (b) Lineal model with lag phase and (c) Sigmoidal model (Garcia et al., 2009)

The most frequently used primary growth models are the non-linear Baranyi, modified Gompertz and the linear model. The primary model developed by Baranyi et al., (1994) describing the colony diameter as a function of time has been used to estimate the, growth rate and lag phase duration. The Baranyi model assumes that after lag phase, growth rate is constant which could lead towards an upper asymptote. The Baranyi model (Eq. 1-2 & 1-3) has been used to describe the change in fungal colony radius as a function of time (Samapundo et al., 2005a, 2005b & 2007c; Astoreca et al., 2012; Marín et al., 2012; Mousa et al., 2013).

$$r = r_0 + \mu_{max}A - \ln\left\{1 + \frac{[\exp(\mu_{max}A)-1]}{\exp(r_{max})}\right\} \quad \text{Eq. 1-2}$$

$$A = t + \left(\frac{1}{\mu_{max}}\right)\ln[\exp(-\mu_{max}t) + \exp(\mu_{max}\lambda) - \exp(-\mu_{max}t - \mu_{max}\lambda)] \quad \text{Eq. 1-3}$$

where r is the colony radius (mm), r_0 initial colony radius (mm, usually zero) μ_{max} is the radial growth rate (non-specific, mm/day), r_{max} is the maximum radius attained, asymptotic value (mm) and λ (days) is the time to growth (lag phase).

The modified Gompertz model that has been used in germination kinetics can also be used in primary modelling. The linear model is the easiest, and it is unlikely that Baranyi or Gombertz could show superiority over the linear correlation (Dantigny et al., 2005a). The trend of the growth curve allows the calculation of two parameters, growth rate (μ, mm/day) of the hyphae which corresponds to the slope of the linear phase and the lag time (λ, day) which is the intercept between regression line and x-axis. Early measurements of colony diameter could improve the accuracy of the estimated lag time since it is derived from extrapolation. Since it is based on the macroscopic observation of mycelium, it has no biological significance. The linear model has been used in assessing the growth parameters of *Aspergillus, Penicillium, Alternaria, Cladosporium, Mucor* and *Eurotium* in different growth substrates (Marín et al., 1995, 1998 and 1999; Velluti et al., 2000; Sautour et al., 2001a, 2001b and 2002; Dantigny et al., 2002 and 2005b; Lopez-Malo et al., 2005).

This method might be considered arbitrary since the data selection is based on the visual inspection of the data set (Dagnas and Membré, 2013).

Marín et al. (2008a), compared the performance of all these three models in fourteen food spoilage fungi. The linear and Baranyi model were found to give the best estimations of growth rate and lag phase when growth did not reach the asymptotic value. The Baranyi model was preferred when there was a decrease in the growth rate. Other methods to assess the growth of the moulds like mycelial weight/biomass, CFU counts and ergosterol content (a fungal cell membrane component) have been tested but remain marginal in use to develop models (Marín et al., 2005).

1.6.2.2. Secondary models

Secondary models relate the kinetic parameters derived from the primary model to describe the influence of environmental factors. Thus, germination or growth parameters (e.g., growth rates, lag phases and decimal reduction rates) are modelled as a function of intrinsic and extrinsic factors of foods, such as temperature and a_w. In contrast to bacteria, a_w has a greater effect on mould development than temperature.

Traditionally, secondary models have been classified into two types depending on the basis of information used to construct the model (McMeekin and Ross, 2002), 1) mechanistic/semi-mechanistic models and 2) empirical models. The mechanistic/semi-mechanistic models are based on understanding the underlying phenomena governing the system, what is actually occurring during growth, giving more insights into the behavior of biological systems (Pitt, 1993). In turn, empirical models simply try to describe the observed responses under the conditions which the experiment was performed (Gibson and Hocking, 1997). Since there is not a clear cut boundary between mechanistic and empirical models, some mechanistic models include aspects of empirical models and vice versa.

1.6.2.2.1. Mechanistic/semi-mechanistic models

Only very few studies are available on the development of mechanistic/semi-mechanistic models describing fungal growth. Thus, predictive mycology remains still a young science in the main stream predictive microbiology. A simple mechanistic model has been used in studying the effect of sorbic acid and pH on the growth of *Penicillium chrysogenum*, *Cladosporium cladosporioides* and *Ulocladium atrum* (Skirdel and Eklund, 1993). A semi-mechanistic model was developed by Pitt (1993), to describe the effects of temperature, pH, a_w and colony size on mould growth and aflatoxin production. The model itself combined 20 different equations and was validated with data from literature.

1.6.2.2.2. Empirical models

Empirical models by virtue of their simplicity and versatility have been the most widely employed models to describe fungal growth. The empirical models used in predictive mycology could be classified as:

- Polynomial models
- Ratkowsky square-root model
- Linear Arrhenius-Davey model
- Rosso cardinal model
- Gamma concept
- Artificial Neural Networks

Polynomial models have been applied more often than the others to describe the fungal growth. These models have been constructed to describe fungal germination and growth as a function of a_w, temperature, pH, $\%O_2$, $\%CO_2$, preservative concentration and their combined effects. General expression is given by (Eq. 1-4),

$$Y = a_0 + \sum_{i=1}^{n} a_i . x_i + \sum_{i=1}^{n} \sum_{i=1}^{n} a_{ij} X_i X_j \qquad \text{Eq. 1-4}$$

where Y is the kinetic response (e.g., growth rate), the coefficients a_0 and a_i are the parameter estimates while X_i and X_j are the variables (e.g., a_w and temperature). Since they are built through multiple linear regression analysis they allow all of the environmental parameters and their interactions to be taken into account. Co-linearity among variables, difficult in sensitive analysis of input variables, over parameterization and the absence of biological meaning of the model parameters are some of the drawbacks in the model (Garcia et al., 2009).

Based on this equation, Gibson et al. (1994) developed the first model to describe the effect of a_w on the growth of *A. flavus, A. oryzae, A. parasiticus* and *A. nomius*. They found that the natural logarithm of μ follows a hyperbolic fitting with b_w. The b_w in the original bacterial model has been replaced with $\sqrt{1 - a_w}$.

$$\ln(\mu_{max}) = a_0 + a_1 . \sqrt{1 - a_w} + a_2 . 1 - a_w \qquad \text{Eq. 1-5}$$

where μ_{max} is the radial growth rate, a_0, a_1 and a_2 are constants to be estimated.
Another polynomial model has been applied by Panagou et al. (2003), to describe the effect of a_w, temperature and pH on *Monascus ruber*. The original linear Arrhenius-Davey equation used for bacterial growth was modified to include the effect of pH.

$$\ln(\mu_{max}) = a_1 + a_2 . pH + a_3 . pH^2 + a_4 . a_w + a_5 . a_w^2 + {a_6}/{T} + {a_7}/{T^2} \qquad \text{Eq. 1-6}$$

where a_1 to a_7 are model parameters and T is the temperature in Kelvin. In all the cases where the model has been applied in fungal growth modelling, the combined effects of the factors are assumed to be additive. Samapundo et al. (2007c) extended the model to include for the first time an interactive term (a_w/T) for the combined effect of a_w and temperature on growth of *A. flavus* and *A. parasiticus*. In addition to growth rate, Baert et al. (2007), showed that the linear Arrhenius-Davey model could be used to predict lag phase in apple puree agar medium (APAM) and apples for *Penicillium expansum*.

Cardinal models were developed because of potential co-linearity among variables and lack of biological meaning in polynomial model constants (Dagnas and Membré, 2013). These models include cardinal values of extrinsic and intrinsic factors (a_w, pH, temperature etc). Generally, three cardinal values are involved for a factor: a minimum (Y_{min}) below which no growth occurs, a maximum (Y_{max}) above which no growth occurs and an optimum (Y_{opt}) where the growth parameter is at its maximal. The first cardinal model developed by Ratkowsky (1983) was extended to include a_w and applied to mould proliferation by Tassou et al. (2007). Moreover, Rosso et al. (1993) proposed a cardinal model with inflection (CMI). Effect of temperature was checked initially but later extended to a_w and pH.

Cardinal models were also used when several factors must be modelled. In these models, no interactive effects are considered and the combined effect is regarded as only multiplicative. The secondary model built based on this approach is the gamma concept .

The general form of the model is given by Eq. 1-7,

$$\gamma = \frac{\mu_{max}}{\mu_{opt}} = \gamma(a_w).\gamma(T).\gamma(pH) \qquad \text{Eq. 1-7}$$

Each gamma term is parameterized so that its value ranges from 0 to 1. This model is highly flexible, allowing the inclusion of large set of mathematical expressions for each gamma term and addition of new gamma terms, whilst relative effects can be calculated by separating the effect of the different variables (Zwietering et al., 1996). However, the applicability of the model is limited in case there are structural correlations between the parameters, which could result in large confidence limits of the parameter values suggesting serious over parameterization of the model (Rosso et al., 1995). Moreover, the model would be unsuitable to use when the interactive effects are additive. The use of the gamma concept in mycology is discussed by Panagou et al. (2003).

Artificial neural networks (ANNs) are highly interconnected network structures consisting of many simple processing elements that can perform many parallel computations for data processing (Hervás et al. 2001). Interest in using ANN in food science is increasing in recent years, as they have shown promising results in several applications such as sensory analysis, pattern recognition,

classification, microbial predictions and food process optimization. Due to their ability to describe highly complex and non-linear problems encountered throughout science, ANNs have been developed more recently as an alternative to conventional regression models (Panagou and Kodogiannis, 2009).

In the field of predictive microbiology, ANNs have been applied to develop models that could estimate growth parameters of bacteria or fungi (García-Gimeno et al., 2002; Panagoue et al., 2007). The use of ANN as an application to fungal growth was very much delayed compared to that of bacteria. Panagou et al. (2007), was the first to apply the ANN to model fungal growth. In this study a radial basis function neural network (RBF NN) was developed to predict the combined effects of temperature, a_w and pH on the radial growth rate of *Monascus ruber* and its accuracy was compared with a quadratic response surface model. Using various statistical indices RBF NN outperformed the quadratic response model using the training data set. Multi-layer perceptron (MLP) ANN and RBF NN were applied to predict OTA concentration over time in grape-based cultures of *Aspergillus carbonarius* under different conditions of temperature, a_w and sub-inhibitory doses of the fungicide carbendazim (Mateo et al., 2009). Coupling of ANN to electronic noses or gas chromatograph-mass spectrometers have found application to classify cereal grains as mouldy or healthy, and to identify spoilage fungi and bacteria (Magan et al., 2003).

1.6.2.3. Tertiary models

Tertiary models could be developed by integrating primary and secondary models in a software using databases. The Pathogen modelling program (PMP, v.7.0) developed by the USDA-ARS (2003), Symprevious, SeafoodSpoilage and Safety Predictor and the latest version of ComBase all were used to predict the bacterial responses. None are related to mould or mycotoxin production (McMeekin et al., 2013).

1.6.3. Probabilistic models

Probabilistic modelling is useful when the purpose is to determine whether or not microbial growth can occur under specific conditions. Especially when mycotoxin-producing species are involved, probabilistic models can be particularly useful as they make it possible to predict whether a particular event, such as growth or toxin production, might occur under specific conditions. Logistic regression is a common tool used to determine growth or no growth (Garcia et al., 2009). Results of previously published studies using probabilistic models indicate that this approach can be satisfactorily applied to predict the combined effect of temperature and a_w on the growth responses of *A. flavus* and *A. parasiticus* (Marín et al., 2012).

The growth/no growth boundary model is another type of model used in predictive mycology. This is a simple model; temporal growth (value 1) or no growth data (value 0) is obtained under certain combinations of extrinsic and intrinsic factors. To obtain a growth/no growth interface, data are fitted to a logistic regression, mostly linked to a polynomial equation.

$$\text{logit}(P) = \ln\left(\frac{P}{1-P}\right) = a_0 + \sum_{i=1}^{n} a_i . x_i + \sum_{i=1}^{n} \sum_{j=1}^{n} a_{ij} X_i X_j \qquad \text{Eq. 1-8}$$

where P is the probability of growth (range 0 to 1), coefficients a_0 and a_i are parameter estimates and the X_i values are the independent factors (e.g., a_w, temperature and pH).

1.6.4. Kinetic predictive models applied to mycotoxin production

Factors which could influence growth like temperature, a_w, inoculum concentration, microbial interactions, physiological state of the mould could also influence mycotoxin contamination. However, the effect of these parameters on mycotoxin formation might be different from that on growth. Moreover, mould growth does not always result in mycotoxin production. The detection of fungi does not necessarily mean that mycotoxins are present and vice versa, absence of fungi does not necessarily mean that there are no mycotoxins. Destruction of mycotoxins by food processing has been difficult since they are highly resistant to heat.

Preventing fungal growth could eventually prevent mycotoxin accumulation. Since growth shows less intraspecific variability and the kinetics of growth are mostly known, the prime approach to prevent accumulation of mycotoxins is through prediction and prevention of mould growth (Marín et al., 2008b). Since poor correlations were observed between growth and mycotoxin accumulation, kinetic models should permit the prediction of conditions where fungus cannot grow at all.

The second approach is the direct mycotoxins analysis and modelling. Hence, the key variable to be modelled will be mycotoxins, instead of mould growth. However, several drawbacks have been associated with this approach (Marín et al., 2008a, 2008b; Garcia et al., 2009) as listed below.

1) Extrapolation of models obtained with one or few strains might not be representative of the majority of the strains, because different fungal strains of the same species have different inherent abilities to produce mycotoxins.
2) Mathematical modelling of myctoxins is particularly difficult due to higher variability in mycotoxin production of a given strain in a given substrate.
3) To develop such models, a large number of analyses for mycotoxins are required, resulting in increased work and costs.
4) It is possible to expect that mycotoxin production follows a curve parallel to growth with slight delay. However, to date the secondary metabolism is poorly understood and the relationship between primary and secondary metabolism is still not clear. Broader generalization about

mycotoxin formation could not be made, thus, the modelling of mycotoxins remains highly challenging.

1.6.4.1. Primary models

Primary models associated to mycotoxin production are quite few at the moment. Mycotoxin production over time has been investigated either in synthetic media or food substrates. Most studies refer to OTA and *Fusarium* toxins, however not taking into account modelling their production (Garcia et al., 2009). OTA production on peanut, maize kernels, dried grapes and coffee beans meal extract agar medium by eight strains of *Aspergillus* section *Nigri* was evaluated at different a_w, temperature and incubation time (Astoreca et al., 2007). DON production *by F. culmorum* and *F. graminearum* in wheat grain over time was studied but not modelled (Hope et al., 2005). Patulin production by *P. expansum* in cold storage apples showed a tendency to increase with time and, it seemed to reach a peak and remain rather constant afterwards (Morales et al., 2007).

A descriptive model for growth and aflatoxin formation affected by environmental conditions was presented by Pitt (1993). Equations were developed for the rates of production and degradation of aflatoxin by *A. flavus* and *A. parasiticus* as related to mould growth and environmental conditions based on the assumption that the rate of toxin formation is proportional to growth rate and cell mass. This is the first one and the only existing mechanistic model in prediction of mycotoxin production (Garcia et al., 2009). Moreover, the different phases of synthesis and degradation of aflatoxin by *A. parasiticus* as a function of time was also studied (Molina and Giannuzzi, 2002).

1.6.4.2. Secondary models

Similarly, secondary models for mycotoxin production are not widespread and they assume constant levels of factors, which is not the case during transport and storage of food products. Bellı´ et al. (2004) modelled OTA production by *Aspergillus* section *nigri* at different a_w levels using multiple linear regression and response surface predictive models. OTA accumulation was modelled using the sigmoidal Gompertz model. However, production of repeatable and reproducible data remained challenging since there was intrinsic biological variability. Moreover, Baert et al. (2007), modelled patulin accumulation as a function of temperature and $\%O_2$ during cold storage time in apple based medium and on apples using a polynomial equation (Eq. 1-9).

$$\text{Patulin concentration (mg/kg)} = a.T + b.S + c.O + d.T.S + e.T.O + f.S.O + g.T.S.O + h.T^2 + i.T^2.S + j.T^2.O + k.T^2.S.O \quad \text{Eq. 1-9}$$

S is the surface (m^2), T is the temperature and O is the oxygen level (%).

Prediction of fungal growth and OTA production by *Aspergillus ochraceus* as influenced by temperature and a_w was studied on irradiated barley grain (Pardo et al., 2004). OTA production was modelled by multiple linear regression and response surface models and provided approximate prediction of growth and OTA production.

In addition, probabilistic models using logistic regression could also be used in modelling mycotoxins (Garcia et al., 2009). OTA production in pistachio nuts by *A. carbonarius* has been modelled (Marín et al., 2008b). The probability for the presence of OTA was correctly predicted in 90% of the cases and reported OTA accumulation was mainly a function of the temperature of storage. In this study, probability models were applied to mycotoxin accumulation for the first time, however, pointed out that further research is required in the kinetics of mycotoxin accumulation to develop proper empirical models. Three equations were developed to predict DON in mature grain at wheat heading, based on rainfall and temperature data, and their timing by Hooker et al. (2002). Prandini et al. (2009) reviewed the predictive models developed for *Fusarium* head blight and related mycotoxin contamination in wheat. Furthermore, Mousa et al. (2011) has modelled aflatoxin production by *A. flavus* isolates in paddy using a second order polynomial equation.

1.6.5. Model validation

Validation of the models is a crucial step after modelling. Generally, internal validation is performed when a new model is developed using the same data used for building up the model (te Giffel and Zwietering, 1999). However, to confirm the robustness of the model an external validation using an independently derived new set of data is essential (Delignette-Muller et al., 1994). Predictive models are often built on data obtained in synthetic media, hence, extrapolation to food products cannot be straight forward due to the complexity of real food matrices. Therefore, a good way of validating a model is to compare its prediction to data obtained in real food matrices.

1.7. CONCLUSIONS

In this chapter a brief introduction on the target spices of this research work is provided. The major classes of mycotoxins, their chemistry, relevant moulds, occurrence and toxicity was briefly explained. Moreover, a comprehensive review on risk assessment with special focus on mycotoxins was given. Finally, the concept of predictive mycology, models applied for growth as well as mycotoxin production were described. The presented literature review could provide the supportive information necessary for better understanding of the research findings discussed in the following chapters.

CHAPTER

2

DEVELOPMENT AND VALIDATION OF A QuEChERS BASED LIQUID CHROMATOGRAPHY TANDEM MASS SPECTROMETRY METHOD FOR THE DETERMINATION OF MULTIPLE MYCOTOXINS IN SPICES

CHAPTER 2: DEVELOPMENT AND VALIDATION OF A QuEChERS BASED LIQUID CHROMATOGRAPHY TANDEM MASS SPECTROMETRY METHOD FOR THE DETERMINATION OF MULTIPLE MYCOTOXINS IN SPICES

Summary

A reliable and rapid method for the determination of multiple mycotoxins was developed using a QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe) based extraction procedure in highly pigmented and complex spice matrices, namely red chilli (*Capsicum annum* L.), black and white pepper (*Piper nigrum* L.). High-performance liquid chromatography tandem mass spectrometry (HPLC-MS/MS) was used for the quantification and confirmation of 17 chemically diverse mycotoxins. Different extraction procedures were studied and optimized in order to obtain better recoveries. Mycotoxins were extracted from the hydrated spices using acidified acetonitrile (1% formic acid), followed by partitioning with NaCl and anhydrous $MgSO_4$; excluding the use of dispersive-solid phase extraction. Significant matrix effect was compensated using the matrix matched calibration curves. Electrospray ionization at positive mode was applied to simultaneously detect all the mycotoxins in a single run time of 20 min. Multiple reaction monitoring mode, choosing at least two abundant fragment ions per analyte was applied. Coefficients of determination obtained were in the range of 0.9844 to 0.9997. Recoveries (ranging from 75 to 117%) were in accordance with the performance criteria required by the European Commission. Inter-day reproducibility ranged from 4 to 22% for most of the mycotoxins. The limit of quantification ranged from 2.3 to 146 µg/kg. The validated method was finally applied to screen mycotoxins in ten samples of each spice matrix. Aflatoxins, ochratoxin, fumonisins, sterigmatocystin and citrinin were among the detected analytes. Positive findings were further confirmed using relative ion intensities. The potentiality of the method to be used for confirmatory purposes according to Commission Decision 2002/657/EC was assessed.

Keywords: *Mycotoxins, spices, QuEChERS, LC-MS/MS, red chilli, pepper*

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2.1. INTRODUCTION

Among various spices, chilli and pepper have been reported as the spices most frequently contaminated with aflatoxins (AFs) and ochratoxins. Most countries have set stringent regulatory requirements on the level of mycotoxins permitted in traded commodities (Van Egmond et al., 2007). According to the latest Commission Regulation No. 165/2010 (EC, 2010a) the stipulated EU maximum level (ML) in spices for AFB1 is 5 µg/kg and 10 µg/kg for total AFs (sum of AFB1, B2, G1 and G2). In addition to AFs, only OTA is currently regulated by EU for spices (*Capsicum* spp., *Piper* spp., nutmeg, ginger, turmeric and mixure containing one or more of these spices). The ML for OTA is 30 µg/kg in *Capsicum* spp. and 15 µg/kg for all other spices (EC, 2010b). As from 2015, a lower ML also for *Capsicum* spp. is foreseen (EC-SANCO, 2012). Meanwhile, maximum AFs levels of 10-20 µg/kg are agreed for the commercial transactions within the international spice trade (Almela et al., 2007). In 2007, the Scientific Committee of the Federal Agency for the Safety of the Food Chain (FASFC) in Belgium decided the necessity for further research into "silent carriers" of mycotoxins like spices, spice extracts and food supplements (FASFC, 2007).

Analysis of mycotoxins is challenging as they are often present at low concentrations in complex matrices. Current analytical methods for the determination of AFs and/or OTA in spices include the use of thin layer chromatography, immuno-affinity chromatography, enzyme linked immuno sorbent assay and high performance liquid chromatography (HPLC) (Santos et al., 2010; Sulyok et al., 2006). To date, several liquid chromatography tandem mass spectrometry (LC-MS/MS) based methods using solid phase extraction (SPE) cleanup are available for multiple mycotoxin analysis for various food commodities (Tanaka et al., 2009; Sulyok et al., 2010; Monbaliu et al., 2009; Njumbe Ediage et al., 2011). However, multi-mycotoxin methods for spices are lacking. Amate et al. (2010) introduced a multi-analyte method for spices, which included pesticide residues, aflatoxins and dyes. Very recently, some existing extraction methods were assessed for multi-residue analysis in paprika and black pepper (Lacina et al., 2012). The aim of the present study was to develop a simple, selective and reliable method based on the QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe) extraction approach for the determination of multiple mycotoxins in spices using LC-MS/MS. Although the QuEChERS method was introduced by the USDA scientists in early 2003 (Anastassiades et al., 2003) for pesticide residue analysis, it has been later extended in the analysis of veterinary drug residues (Stubbings and Bigwood, 2009), antibiotics (Lombardo-Agüí et al., 2012), acrylamide (Mastovska and Lehotay, 2006) and mycotoxins (Tamura et al., 2011; Sirhan et al., 2011; Cunha and Fernandes, 2010; Rasmussen et al., 2010) in different matrices. To our knowledge this is the first study to develop a QuEChERS method for the quantitative determination of multiple mycotoxins in spices using LC-MS/MS.

2.2. MATERIALS AND METHODOLOGY

2.2.1. Chemicals and reagents

LC-MS grade absolute methanol (MeOH) and analytical grade acetonitrile (MeCN) were purchased from VWR International (Zaventem, Belgium). Formic acid ULC-MS grade (99%) was supplied by Bio Solve B.V. Ammonium formate (±99%) was obtained from Sigma-Aldrich, Steinheim. Formic acid analytical grade (98-100%) and sodium chloride (±99.5%) were from Merck (Darmstadt, Germany). Magnesium sulphate anhydrous (±99%) was purchased from nacalai tesque Inc. (Gentaur; Kyoto, Japan). Ultrafree®-MC centrifugal filter devices (0.22 µm) were obtained from Millipore (Bredford, MA, USA). Water was purified (18 MΩ) on a Milli-Q Plus apparatus (Millipore; Brussels, Belgium). All other chemicals and reagents used were of analytical grade.

2.2.2. Mycotoxin standards

Mycotoxin reference standards (standards with known concentration, but without certification) namely, deoxynivalenol (DON), 3-acetyldeoxynivalenol (3-ADON), 15-acetyldeoxynivalenol (15-ADON), neosolaniol (NEO), aflatoxin B1 (AFB1), aflatoxin B2 (AFB2), aflatoxin G1 (AFG1), aflatoxin G2 (AFG2), ochratoxin A (OTA), fumonisin B1 (FB1), fumonisin B2 (FB2), HT-2 toxin (HT-2), alternariol methyl ether (AME), zearalenone (ZEN), sterigmatocystin (STERIG) and zearalanone (ZAN) were purchased from Sigma-Aldrich (Bornem, Belgium). NEO was obtained as solution (100 µg/mL) in MeCN. T-2 toxin (T-2) was purchased from Biopure (Tulln, Austria). Fumonisin B3 (FB3) was supplied by Promec Unit (Tygerberg, South Africa). Roquefortine C (ROQ C) was purchased from Enzo Life Science (Lorrach, Germany). FB2 and FB3 standards at a concentration of 1 mg/mL were prepared in MeCN/water (50/50, v/v). Stock solutions of DON, 3-ADON, 15-ADON, AFB1, AFB2, AFG1, AFG2, OTA, FB1, HT-2, T-2, ZEN, STERIG, ZAN and ROQ C were prepared in MeOH at a concentration of 1 mg/mL. Stock solution of AME (1 mg/mL) was prepared in MeOH/dimethylformamide (60/40, v/v). All the stock solutions were stored for maximum one year at (-20)^{o}C except FB2 and FB3 which were stored at 4^{o}C.

From the individual stock standard solutions, working solutions were prepared by diluting them in MeOH. A standard mixture of mycotoxins was prepared using the individual stock and working standard solutions at the following concentrations: AFB1, AFB2, AFG1 and AFG2 (0.5 µg/mL), OTA and ROQ C (1.0 µg/mL), STERIG (0.625 µg/mL), T-2, HT-2, NEO, 3-ADON and 15-ADON (2.5 µg/mL), DON, FB1, FB2, FB3, AME and CIT (5 µg/mL). The standard mixtures were prepared in MeOH, stored at (-20)^{o}C and renewed every 2 months. Necessary precautions were taken to avoid photo-degradation of the light sensitive mycotoxins, such as wrapping the standard solutions and the extracts with aluminum foil and by storing them in dark.

2.2.3. Samples

The spice samples of black pepper, white pepper and red chilli were collected in random from Sri Lankan markets during 2011-2012, Jan-Mar (200-500 g). Different forms of spices include whole pepper, crushed pepper, pepper powder, whole chilli, chilli flakes and chilli powder. The samples were packed air-tight in low density poly ethylene (LDPE) and transferred to Belgium. Samples were stored at room temperature until analysis. Ten samples of each spice matrix were used to evaluate the applicability of the developed method.

2.2.4. Sample preparation

Samples were extracted using a modified QuEChERS based approach. A very simple and straightforward extraction procedure was applied. All the different forms of spices were finely ground using a universal mill (grinder) (M20 IKA®-WERKE; Staufen, Germany). Finely ground and homogenized spice sample (the sample was powdered and it was not sieved as there were no coarse particles observed; homogenity assumed based on grinding and mixing, no specific homogenity test was done) of 1.00±0.05 g was weighed in a 50 mL extraction tube. The sample was spiked with a mycotoxins standard mixture containing standard mycotoxins at different concentrations. After leaving the samples for an hour for equilibration, 5 mL water was added and mixed with a vortex for 1 min. Samples were left for soaking for further 30 min. Thereafter, 5 mL of the extraction solvent (MeCN/1% formic acid v/v) was added and after a brief shaking, samples were extracted using an end-over-end shaker (Agitelec, J. Toulemonde and Cie, Paris, France) for 20 min. Subsequently, 2.00±0.05 g of the pre-weighed $MgSO_4$ anhydrous salt and 0.50±0.01 g of NaCl were added and the tube was capped immediately (a brief hand shaking immediately after the addition of salts was performed to prevent agglomeration of the salts). The tubes were then vortexed for 2 min and centrifuged at 4000x g for 7 min. Shaking and centrifugation was carried out in order to induce phase separation and mycotoxins partitioning. Finally, an aliquot of the supernatant MeCN layer was subjected for centrifuge filtration at 10,000x g for 3 min. After filtration an aliquot was transferred to the vials for LC-MS/MS analysis.

2.2.5. Instrumental conditions

2.2.5.1 HPLC apparatus and conditions

Liquid chromatography was performed using a waters ACQUITY ultra-performance liquid chromatography (UPLC™) system. The analytical column used was a Symmetry C18, 5 µm, 150 x 2.1 mm (Waters; Zellik, Belgium) and the guard column was a Sentry, 3.5 µm, 10 x 2.1 mm (Waters; Zellik, Belgium), with a flow rate of 0.3 mL/min. An aliquot of 10 µL sample extract was injected into the chromatographic system. The partial loop mode was used as an injection technique. Volumes of weak wash (10% MeOH) and strong wash (100% MeOH) solvents were 500 µL each.

The column and sample temperature were maintained at room temperature and 10°C, respectively. Mobile phase A was MeOH/water (20/80 v/v) and mobile phase B was MeOH/water (90/10 v/v), both contained 5mM ammonium formate and 0.1% formic acid. A gradient elution programme starting with 50% B was maintained for 2 min. From 2 to 10 min it linearly increased to 100% B. Over further 5 min, the gradient was kept unchanged at 100% B. In 1 min the gradient switched to 50% B and was equilibrated at the initial mobile phase conditions for further 4 min before the start of next injection. Total run time was 20 min.

2.2.5.2. MS/MS apparatus and conditions

Mass spectrometry (MS/MS) was performed with a Quattro Premier^{TM} XE tandem quadrupole mass spectrometer (Waters; Milford, MA, USA). The MS was operated at electrospray ionization in positive mode (ESI+). For infusion experiments, 10 ng/µL of the mycotoxin standard dissolved in mobile phase B were used at a flow rate of 10 µL/min (confirmed similar fragment ions formation with the in-house developed multi-mycotoxin method (Monbaliu et al., 2009); there were no major changes on fragmentation ions type observed despite the differences in the injection solvent used). The capillary voltage was set at 3.5 kV. Nitrogen was used as cone, nebulizing and desolvation gas. Cone voltage was defined for each analyte separately (Table 2-1). Extractor cone voltage (V) was 3. Source temperature was 350°C. The desolvation temperature was set at 130°C. The cone and desolvation gas flow were maintained at 50 L/h and 800 L/h, respectively. First quadrupole settings (Q1): low mass resolution (LM1) was 14, high mass resolution (HM1) was 14 and ion energy 1 was 0.1. Collision cell settings (Q2): entrance was -1 and exit 0.0. Third quadrupole settings (Q3): low mass resolution (LM2) was 13.5, high mass resolution (HM2) was 13.5 and ion energy 2 was 1.5. Multiplier voltage was 650 V. Collision gas flow was set at 0.2 mL/min. Analysis of the mycotoxins was performed in multiple reaction monitoring (MRM) mode. For each mycotoxin, at least one precursor ion and two fragment/product ions were monitored. The most abundant product ion was selected for quantification and the second intense one for qualification. The quantification and qualification ion transitions of the respective mycotoxins and the optimum collision energies (collision energy 1 and collision energy 2) and cone voltages were programmed (Table 2-1). For data acquisition and processing, Masslynx and Quanlynx software 4.0 (Waters) were used.

2.2.6. Matrix effect evaluation

The matrix effect (ME) was evaluated by comparing the peak responses of the standard mycotoxins (n=3) spiked in the extraction solvent with the spiked spice extracts at six concentration levels for each analyte. A standard mixture of mycotoxins was prepared using the individual stock and working standard solutions at the following concentrations for determining the ME: AFB1, AFB2,

AFG1 and AFG2 (0.5 µg/mL), OTA and ROQ C (1.0 µg/mL), STERIG (0.625 µg/mL), T-2, HT-2, NEO, 3-ADON, 15-ADON, DON, FB1, FB2, FB3, AME and CIT (2.5 µg/mL).

Table 2-1. Parameters for the mass spectrometric detection of mycotoxins including analyte retention time (tR), precursor ions, pseudo-molecular ion, cone voltage, quantification ions (Quan), qualification ions (Qual), collision energy 1 (CE1) and collision energy 2 (CE2).

Mycotoxin	tR (min)	Precursor ion (m/z)	Pseudo-molecular ion	Cone voltage (V)	Quan (m/z)	Qual (m/z)	CE1 (eV)	CE2 (eV)
AFG2	2.23	331.0	$[M+H]^+$	53	313.1	245.2	30	25
AFG1	2.50	329.0	$[M+H]^+$	45	243.0	311.2	25	20
AFB1	3.23	313.0	$[M+H]^+$	51	285.1	241.2	24	36
AFB2	2.88	315.0	$[M+H]^+$	51	287.2	259.2	27	30
OTA	7.41	403.9	$[M+H]^+$	25	239.0	358.2	22	20
T-2	6.09	484.1	$[M+NH_4]^+$	30	215.0	185.1	20	18
HT-2	5.03	442.2	$[M+NH_4]^+$	20	263.1	215.0	13	13
STERIG	7.96	325.0	$[M+H]^+$	47	310.2	281.1	25	36
ROQ C	4.33	390.0	$[M+H]^+$	40	193.2	322.2	26	21
FB1	5.28	722.4	$[M+H]^+$	56	704.4	352.4	29	36
FB2	7.55	706.0	$[M+H]^+$	50	336.5	318.0	35	29
FB3	6.55	706.1	$[M+H]^+$	54	688.5	354.0	34	31
CIT	5.63	250.9	$[M+H]^+$	32	233.2	205.4	17	26
AME	8.74	272.9	$[M+H]^+$	57	258.2	199.3	26	30
3-ADON	2.26	339.2	$[M+H]^+$	24	231.2	261.4	12	10
15-ADON	2.26	339.2	$[M+H]^+$	24	137.2	203.2	10	12
NEO	1.64	400.1	$[M+NH_4]^+$	26	185.0	305.3	19	12
DON	1.58	297.1	$[M+H]^+$	26	249.2	231.2	15	10
ZAN (IS)	7.23	321.0	$[M+H]^+$	27	303.3	189.2	13	19

To prepare the spice extracts with AFs concentrations 5, 10, 20, 40, 100 and 150 µg/L, following volumes of the spice extracts 495, 490, 240, 230, 200 and 175 µL were spiked with 5, 10, 10, 20, 50, 75 µL of the standard mixture, respectively. The ME was calculated via the formula: ME (%) = (A2-A1/A1)*100, where A1 is the average area of the mycotoxin standard in solvent (MeCN/formic

acid (99/1 v/v)) at a specific concentration and A2 is the average area of the mycotoxin standard in blank spice extract at the same concentration (Chambers et al., 2007). In this way it was possible to compare the positive or negative ME, that is an increase or decrease of the detector response, respectively.

2.2.7. Method validation study

The multi-mycotoxin analytical method optimized for the three different spices was validated using spiked blank spice samples. Several samples were analysed in advance to obtain a sample that is free of analyte at the particular retention time (tR) of the analyte. We were able to get the blank samples for both peppers. However, for red chilli it was rather difficult to get a Sri Lankan sample free from AFB1. Matrix components of the spices may differ between regions hence, to resemble the similar matrix complexity the selected Lankan chilli sample was preferred to use throughout the validation study, with the blank subtraction for AFB1. A set of performance characteristics that were in compliance with the recommendations and guidelines defined by the Commission Decision 2002/657/EC (EC, 2002) and Regulation EC/401/2006 (EC, 2006a) were evaluated. Validation parameters assessed were, linearity, recovery, limit of detection (LOD), limit of quantification (LOQ), repeatability (intra-day precision; RSDr), reproducibility (inter-day precision; RSDR) and specificity.

2.2.7.1. Calibration curves, linearity, LOD, LOQ and recovery

Linearity was evaluated using matrix matched calibration (MMC) curves, by spiking blank samples at six concentration levels for the three different spice matrices. Peak area was used as analyte response. Calibration curves were constructed by plotting the peak areas (y) versus the concentration of analytes (x). The concentration ranges used for this study were*:* AFs (5-40 µg/kg); OTA and ROQ C (10-80 µg/kg); T-2, HT-2, NEO, 3-ADON and 15-ADON (50-250 µg/kg), STERIG (6.25-75 µg/kg), FB1, FB2, FB3, AME and CIT (100-600 µg/kg). Calculations were performed on the average peak areas (n=6); relative standard deviations (RSDs), calibration curve equations and the determination coefficients (R^2) for each mycotoxin were determined.

LOD and LOQ were determined using the MMC curves. LODs were determined as the concentration corresponding to three times the standard error of the y-intercept divided by the slope. The linest function of the Microsoft excel 2010 programme was used. LOQ equaled the concentration corresponding to six times the standard error of the y-intercept divided by the slope; which is two times the LOD. For each of the analyte, the calculated LODs and LOQs were also verified by the S/N ratio which should be more than 3 and 10 (Vial and Jardy, 1999). The validation

experiments that were used to calculate the LODs and LOQs were utilized also to calculate the recovery of the method. International Union of Pure and Applied Chemistry (IUPAC) defines the apparent recovery as the ratio of the predicted value obtained from the MMC curves divided by the actual/theoretical value (Sulyok et al., 2006).

2.2.7.2. Intra-day repeatability, inter-day reproducibility and specificity

Precision of the method was assessed by repeatability and inter-day reproducibility (within laboratory) experiments. Intra-day repeatability of the method was evaluated by spiking the mycotoxins standard solutions to the blank spice matrices at four different concentration levels (n=6) and analyzing in the same run of the day on the LC-MS/MS. Inter-day reproducibility of the method was determined by repeating this experiment consecutively for three different days for all the spice matrices. Specificity of the method was performed by analyzing the blank samples and matrix interferences were checked close to the elution zone of each analyte.

2.3. RESULTS AND DISCUSSION

2.3.1. Method development

2.3.1.1. Extraction solvent selection and evaluation of cleanup

In multiclass mycotoxin methods, the most critical step is the optimization of the extraction and clean up procedure, especially for complex matrices such as spices, which contain flavonoids, terpenes and alkaloids (Amate et al., 2010). Following extraction solvent combinations (v/v) were initially investigated for achieving acceptable recoveries for each analyte from different matrices: MeCN (100%), MeCN/acetic acid (AA) (99/1), MeCN/water/AA (79/20/1) (Sulyok et al., 2006), MeOH/water/AA (79/20/1), MeOH/MeCN/AA (79/20/1), MeOH/ethyl acetate/water (70/25/5). Prior to any solvent extraction, the matrices were soaked in 5 mL water. Better liquid-liquid partitioning with salts was only obtained with the solvent combinations containing MeCN. Due to the absence of salt-induced partitioning with MeOH containing solvents, the extracts obtained were too dark in colour compared to the MeCN extract. When using pure MeCN as the extraction solvent, only AFB1, AFB2, AFG1, AFG2, OTA, T-2, HT-2, STERIG and ROQ C could be detected in the red chilli matrices and only AFs, T-2 and HT-2 could be recovered in both pepper matrices. In addition to the poor extraction of many other toxins in peppers, fumonisins recovery was very poor in both matrices. Addition of 1% AA (MeCN 99/1 v/v) to the extraction solvent increased the recovery of all the above said mycotoxins, but the sensitivity was low. Further, the apparent recovery for OTA (40 µg/kg) in both pepper matrices exceeded the acceptable range (142%) specified under EC performance criteria (EC, 2006a). Instead of AA, addition of 1% formic acid to MeCN (99/1 v/v) therefore was investigated on toxins extraction and chromatographic

performances. Inclusion of formic acid helped in better recovery of fumonisins (FB1, FB2 and FB3) with improved peak responses compared to AA in the solvent. Significant increase in fumonisins response was obtained for red chilli compared to both peppers. Moreover, peak responses for all the AFs and STERIG were significantly higher in all the three spices with the addition of FA. However, the responses were lower in case of HT-2 in all the spices, OTA in both peppers and T-2 in black pepper with this solvent combination. Since spices were soaked in water in the beginning, addition of water to the solvent mixture was eluded.

Additionally, the extraction efficiency of DON, 3-ADON, 15-ADON, AME and CIT were assessed using this solvent. Extraction of DON was found not reproducible in all the matrices, so only qualitative identification was possible. DON is highly polar so separation of the aqueous phase probably had a negative effect on its extraction. However, comparatively better results were obtained with the DON derivatives in all the spices studied, possibly due to their slightly lower polarity. AME was only extractable from red chilli. Strong ion suppressions for AME were observed in pepper matrices even at very high spiking concentration. Peaks for CIT were more uniform in chilli than in peppers. Since addition of formic acid helped to extract most toxins the solvent combination MeCN/formic acid (99/1 v/v) was selected as the best solvent of compromise for the extraction of mycotoxins in all the three spices.

Since the QuEChERS method was initially developed for fruits and vegetables which contain plenty of water, it is generally recommended to add water in the beginning to dry food products. Therefore, water was added to dry spices in order to hydrate them prior to extraction. Soaking the spice in water could help to swell the matrix and weaken the interactions of the analyte with the matrix components and assist in efficient extraction. Different volumes of water to extraction solvent combinations were investigated (10/15, 10/10, 10/5, 5/15, 5/10, 5/5 v/v mL) per 1 g of the spice matrix using the same amount of salts. The water to solvent ratio is identical for 10/10, 5/5 v/v mL. Salt partitioning was observed in all the cases. The aliquot from the total extracted solvent volume was tested. A compariso+n of peak responses between the 10/15 and 5/5 ratio is shown in Fig. 2-1. Highest responses were observed with 5/5 (v/v mL) water to solvent ratio for most of the analytes although these were comparable to the response obtained in 10/5 (v/v mL) for AFG2, AFG1, AFB2, T-2 and ROQ C. Reduced water to solvent ratio helped to detect mycotoxins at lower concentrations (5-10 µg/kg of aflatoxins and OTA) with acceptable recoveries (72-121%) in all the spice matrices.

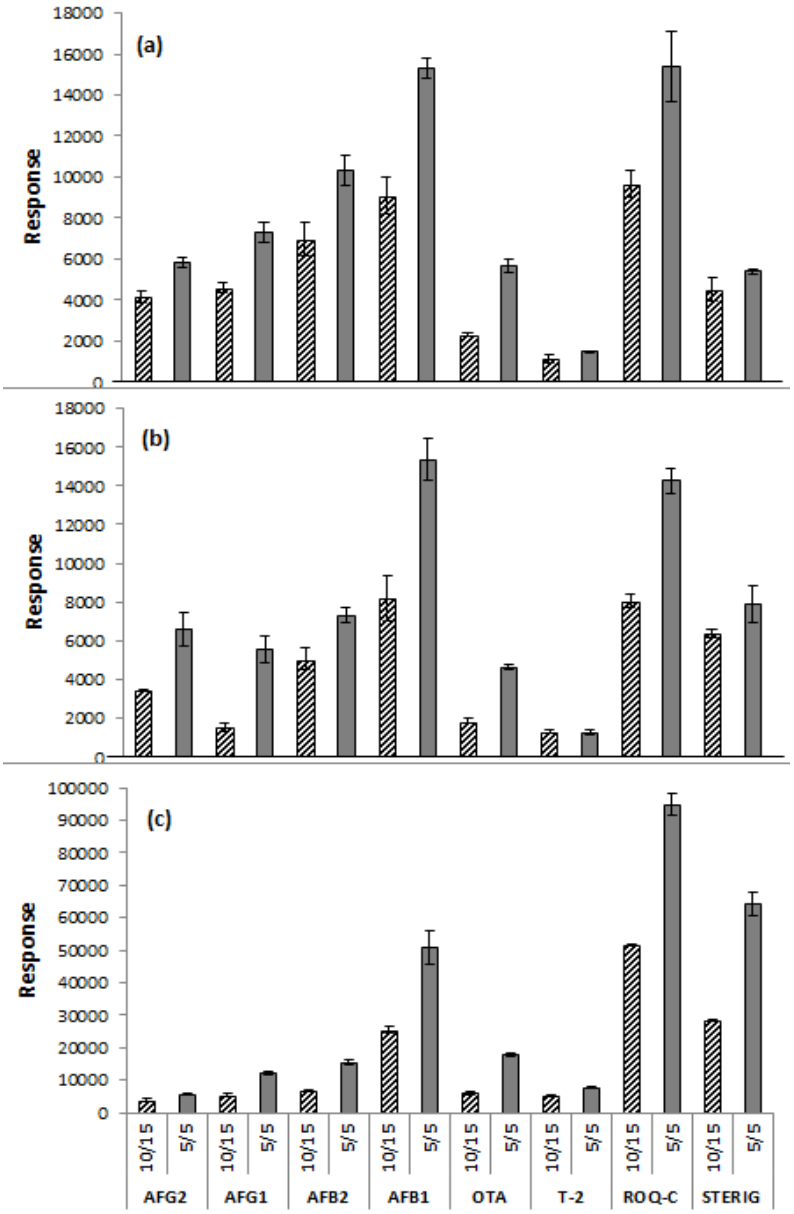

Fig. 2-1. Peak responses obtained at one concentration level for different mycotoxins with two different water to solvent ratios (10/15 and 5/5 v/v mL): (a) white pepper, (b) black pepper and (c) red chilli.

2.3.1.2. Effect of freezing out and decolourization

Concentrating the analyte in order to increase the sensitivity was evaluated. However, this resulted in dark and turbid residues probably because of the co-extracted etheric oils. Application of a freezing out step resulted in increased responses but only for some toxins, T-2 (36%) in chilli, AFG2 (93%), T-2 (37%) and STERIG (44%) in black pepper and AFG1 (67%) and STERIG (46%) in white pepper hence, the extra analytical time was found not worth. The conventional QuEChERS implying a dispersive-SPE (*d*-SPE) cleanup step, using adsorbents like graphitized carbon black (GCB) or primary secondary amine (PSA) as sorbent materials was also investigated.

GCB (5 mg/mL extract) removed almost all of the pigments and produced very clear extract, but very poor signals were obtained with most of the analytes. PSA (50 mg/mL) produced a clear extract with black pepper and red chilli, but further evaluation was ignored since its nature (amino group) to bind the fumonisins (carboxylic acid) and influence on fumonisins recovery (Zachariasova et al., 2010). Zinc acetate (125 mg/mL) removed pigments, but the recovery of aflatoxins were affected. Although the pigments could not be completely removed with salt partitioning only, it resulted in a clear and transparent extract. Chromatograms of four different mycotoxins (OTA, T-2, ROQ C and STERIG) obtained in white pepper with and without salt addition is shown in Fig. 2-2. Finally, it was decided to continue the extraction as described in section 2.2.4. The life time of the column was not affected by this extract.

In most of the QuEChERS based mycotoxin analytical methods ammonium formate was used (Frenich et al., 2011; Rasmussen et al., 2010; Zachariasova et al., 2010) as mobile phase additive. In the initial stages of method development the mobile phases A: 80/20 water/MeOH (v/v) and B: 90/10 MeOH/water (v/v) both containing 5mM ammonium formate were assessed.

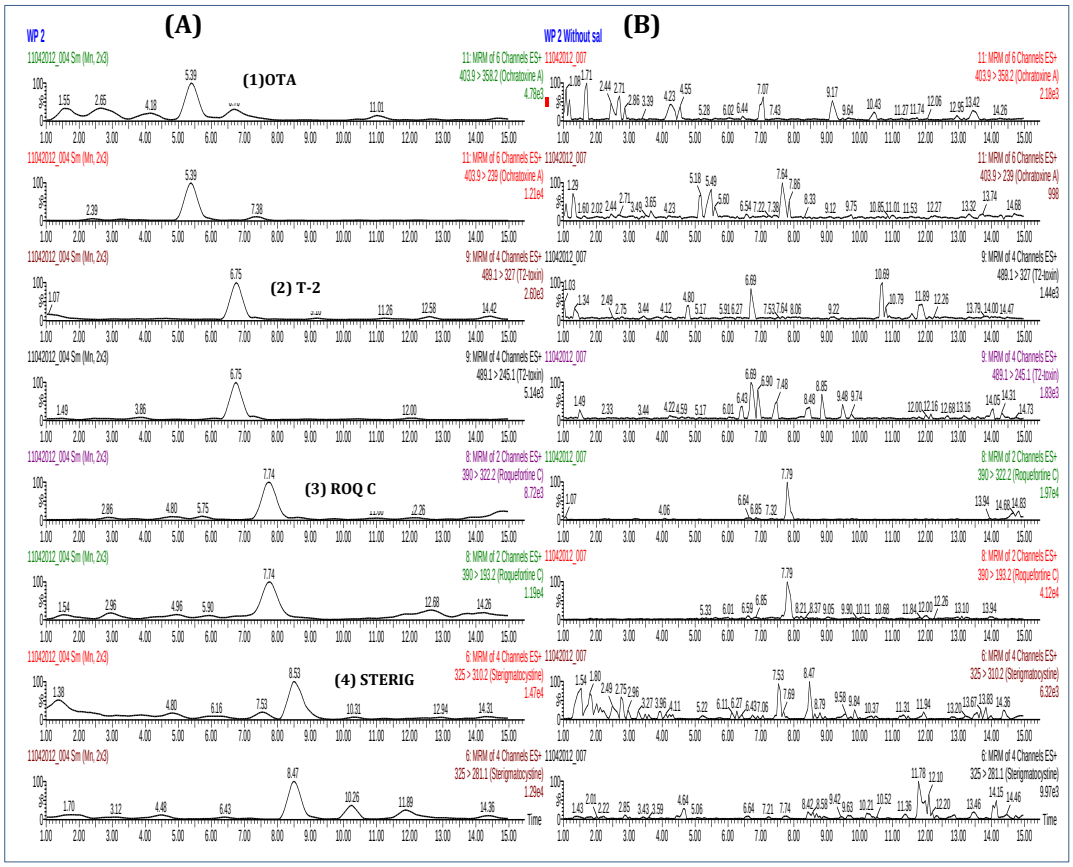

Fig. 2-2. MRM chromatograms obtained in white pepper: (A) with and (B) without salt partitioning for (1) OTA, (2) T-2, (3) ROQ C and (4) STERIG at 100 µg/kg.

2.3.1.3. Optimization of the chromatographic conditions

Compared to other mycotoxins in this study, ionization of the fumonisins was lower in all the matrices. Since fumonisins are highly ionic, having four tri-carboxylic groups in their molecular

structure it is reported that acidic chromatographic conditions could improve their ionization (Sulyok et al., 2010). Hence, addition of formic acid to the mobile phases was evaluated on the chromatographic performances of each analyte. With the slight acidification of mobile phase, significant increase in ionization was obtained for most of the mycotoxins, except OTA (Fig. 2-3). In addition to the ionization intensity, peak uniformity was also improved with acidified mobile phase. Hence, mobile phases with 0.1% formic acid were selected. All the mycotoxins were eluted with good selectivity and MS sensitivity in a gradient run time of 10 min. Cleaning and re-equilibration steps included further 10 min. The order of peak elution of all examined mycotoxins is shown in a chromatogram obtained with red chilli (Fig. 2-4). Except 3-ADON and 15-ADON, all other mycotoxins have shown good peak resolution. The overlay of 3- and 15-ADON is a compromise in our method. Since neither of these compounds were found during the screening of spices, so less importance was given for the separation of these acetyl derivatives. Applying UPLC conditions on Acquity UPLC HSS T3 column was used to seperate these ADONs (Han et al. 2010).

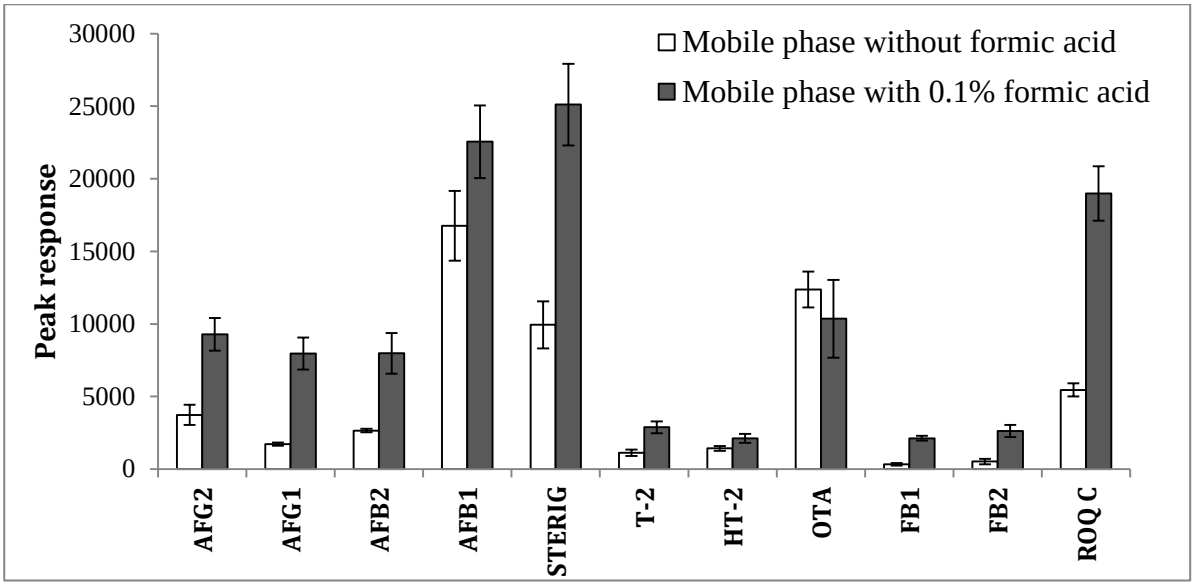

Fig. 2-3. Peak responses obtained in red chilli (n=3), without and with the addition of formic acid in mobile phases (AFG2, AFG1, AFB2 and AFB1 at 20 µg/kg, STERIG at 25 µg/kg, T-2 and HT-2 at 50 µg/kg, OTA and ROQ C at 20 µg/kg, FB1, FB2 and FB3 at 80 µg/kg; other mycotoxins were not tested).

Selection of the MS conditions was initially based on the in-house developed multi-mycotoxin method (Monbaliu et al., 2009), but other multi-mycotoxin methods developed on different matrices (Frenich et al., 2011; Tanaka et al., 2009; Sulyok et al., 2010; Sulyok et al., 2006; Njumbe Ediage et al., 2011; Di Mavungu et al., 2009; Tamura et al., 2011; Sirhan et al., 2011; Cunha amd Fernandes, 2010; Rasmussen et al., 2011) were referred to adjust the MS conditions and select different MRM transitions during the course of the study. Optimized MS conditions are shown in Table 2-1. The

fragment ions reported in other multi-mycotoxin analytical methods were investigated in our spice matrices. In some occasions more than two fragment ion transitions were programmed in the MRM for additional confirmation, as strong ME was encountered in spices. Apart from the selection of two fragment ions, the relative ion intensity (peak area secondary ion/peak area primary ion*100) of the two transitions was additionally assessed to meet the identification criteria (EC, 2002). The relative ion intensities of the standards were compared with that of matrix samples. MRM chromatograms obtained with a standard mycotoxin mixture are shown in Fig. 2-5. At positive ESI, protonated molecular ions $[M+H]^+$ were formed as precursor ions for most of the analytes. In the case of NEO, T-2 and HT-2, ammonium adducts $[M+NH_4]^+$ were formed as precursor ions. Ammonium adducts formation of these type A-trichothecenes was due to the ester groups in their structure. Intensive ammonium adducts were formed only for those molecules with ester groups at both C15 and C4 positions (Razzazi-Fazeli et al., 2002). Seems there is a higher tendency to produce ammonium adducts if there are ester groups in both these positions. However, ester group at C15 could also produce ammonium adducts as it was observed with HT2. Berger et al. (1999) have reported strong ammonium adducts in all cases of trichothecenes with an acetyl group at C15.

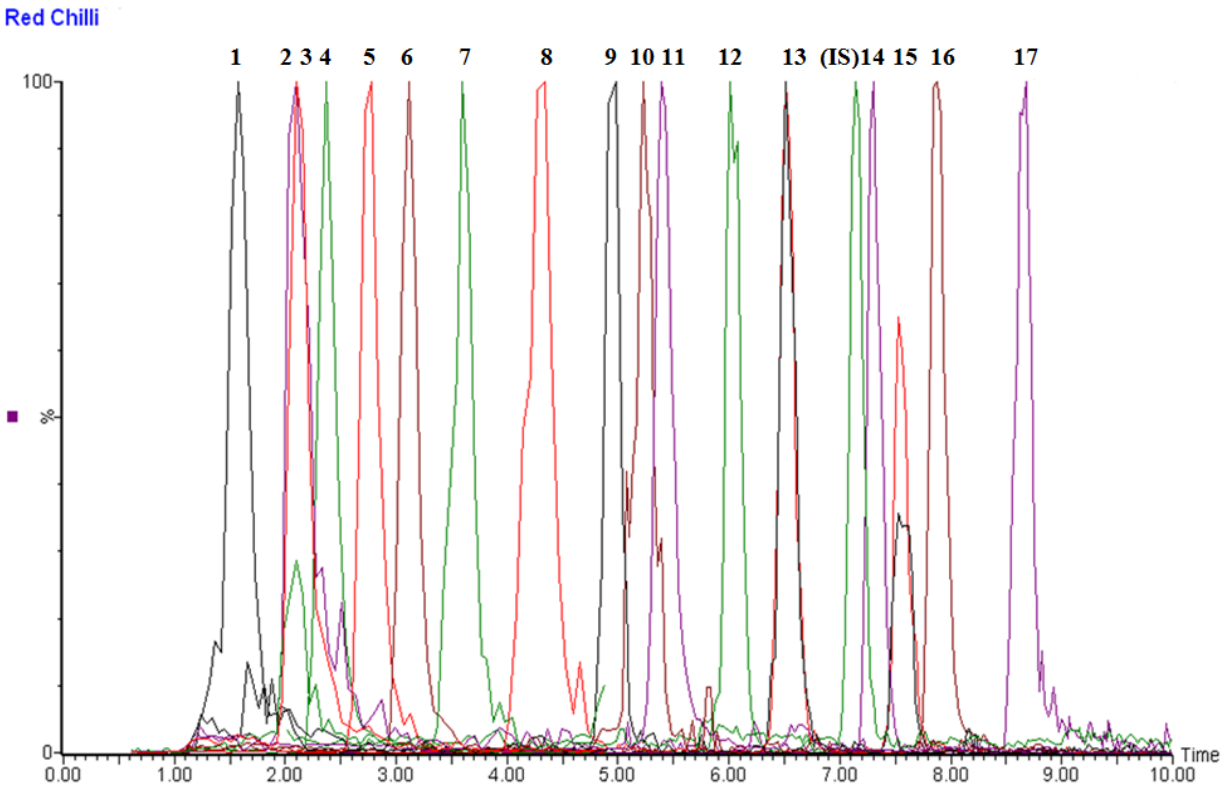

Fig. 2-4. Chromatograms showing order of peak elution of mycotoxins in red chilli matrix (normalized chromatograms). 1) NEO, 2) 3-ADON, 3) 15-ADON, 4) AFG2, 5) AFG1, 6) AFB2, 7) AFB1, 8) ROQ C, 9) HT-2, 10) CIT, 11) FB1, 12) T-2, 13) FB3, IS) ZAN, 14) OTA, 15) FB2, 16) STERIG and 17) AME.

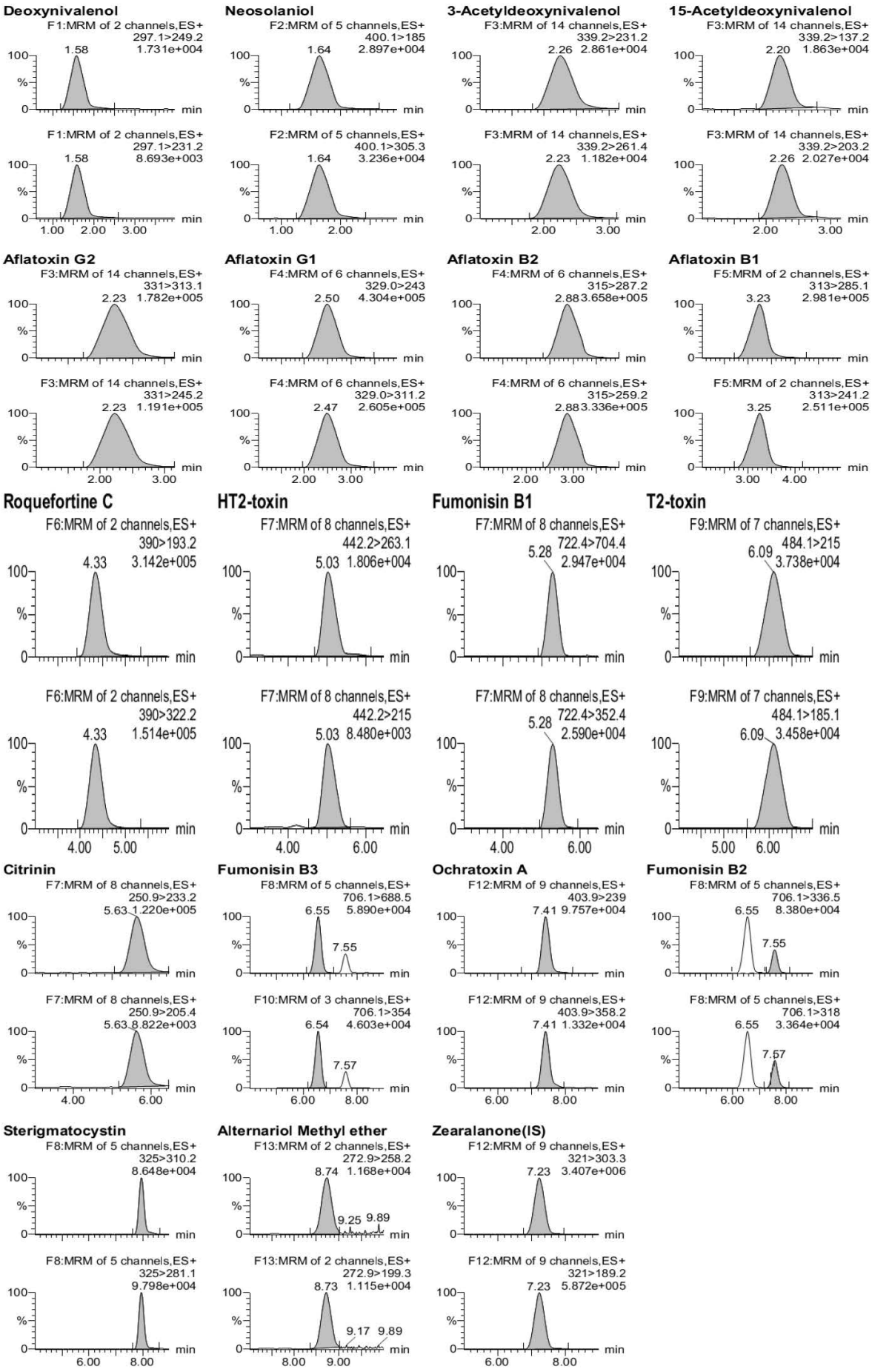

Fig. 2-5. MRM transitions, quantification and qualification ions obtained with a mycotoxin standard mixture: AFs at 12.5 µg/kg, STERIG at 15.625 µg/kg OTA and ROQ C at 25 µg/kg, T-2, HT-2, 3-ADON, 15-ADON and NEO at 62.5 µg/kg and all other toxins at 125 µg/kg.

The formation of AFB2 fragment ions m/z 287.2 and 259.2 could be similar as explained by Liau et al. (2007). Fragment ion m/z 241.2 of AFB1 formation can be explained by the removal of $-C_3H_4O_2$ from the precursor ion. The loss of group $-C_3H_2O_3$ from the AFG1 precursor ion will lead to the formation of the fragment m/z 243. The lactone ring is more prone to fragmentation than the difuranic ring in all the aflatoxins studied. The fragment ions formation from OTA (m/z 239, 358.2 and 221) could be postulated as similar to Lau et al. (2000). These ions were also reported in previous studies with similar order of intensity, regardless of the different ionization conditions used.

The most intense fragment ion of FB1 (m/z 704.4) was due to the loss of a water molecule $[M+H-H_2O]^+$, while the qualification ion (m/z 352.4) corresponds to the elimination of two tri-carboxylic acid (TCA) from the side chains and a concomitant loss of a water molecule $[M+H-2TCA-H_2O]^+$. Moreover, the fragment ion m/z 334.4 was also observed due to an extra elimination of a water molecule (Josephs, 1996), which corresponds to the hydroxyl group of the fumonisin backbone. However, latter ion (m/z 334.4) was reported as the quantitation ion for FB1 in several other multi-mycotoxin methods (Di Mavungu et al., 2009; Tamura et al. 2011; Rasmussen et al., 2010; Zachariasova et al., 2010) in contrast to our observation. The FB1 fragment ion m/z 528 $[M+H-H_2O-TCA]^+$) was selected as the confirmation ion in QuEChERS extract of maize silage (Rasmussen et al., 2010), was only the fourth largest intense ion in our conditions.

Both FB2 and FB3, the structural analogues of FB_1 produced similar fragment ions (both 16 amu lower than the corresponding FB1 fragments). Both of these analytes have been reported to produce following fragments: m/z 688.5 $[M+H-H_2O]^+$, m/z 354 $[M+H-2TCA]^+$, m/z 336.5 $[M+H-H2O-2TCA]^+$ and m/z 318 $[M+H-2H2O-2TCA]^+$. Therefore, double peaks were observed for both of these analytes in their respective MRM chromatograms. However, they were separated by finding these different fragment ions corresponding to their tR. FB1 is more polar than FB2 and FB3, as it has one more hydroxyl group, thus it has the lowest tR. Though, the structurally related FB2 and FB3 having the same molecular weight, the elution of FB3 occurs a minute ahead of FB2 because the polarity of FB3 is higher than FB2 as it could be assessed from the position of –OH group in their structures (Tamura et al., 2011). In FB3 it is close to the electron dense TCA moiety hence, it will tend to influence more the overall polarity of the molecule in contrast to its presence close to the alkyl group as on FB2. Hence, an elution order of FB1, FB3 and finally FB2 was obtained (Fig. 2-4). The fragment ions of CIT, m/z 233.2 and m/z 205.4 could be due to the dismissal of a water molecule and -COOH group, respectively. Fragmentation of AME could be proposed in the following way, m/z 258.2 $[M+H-H_2O]^+$ and m/z 199.3 $[M+H-CH_3-OCH_3-CO]^+$.

2.3.2. Evaluation of matrix effect

Matrix effects are common problems that occur when using LC-MS or MS/MS, and thus have an adverse effect on the analytical results. The response of the target compound can be enhanced or suppressed due to the interfering matrix components, which is commonly known as signal suppression/enhancement effect (SSE). The ME of different spices on different analytes is shown (Fig. 2-6). It can be seen that the signal suppression effect was very prominent for 75% of the analytes in pepper.

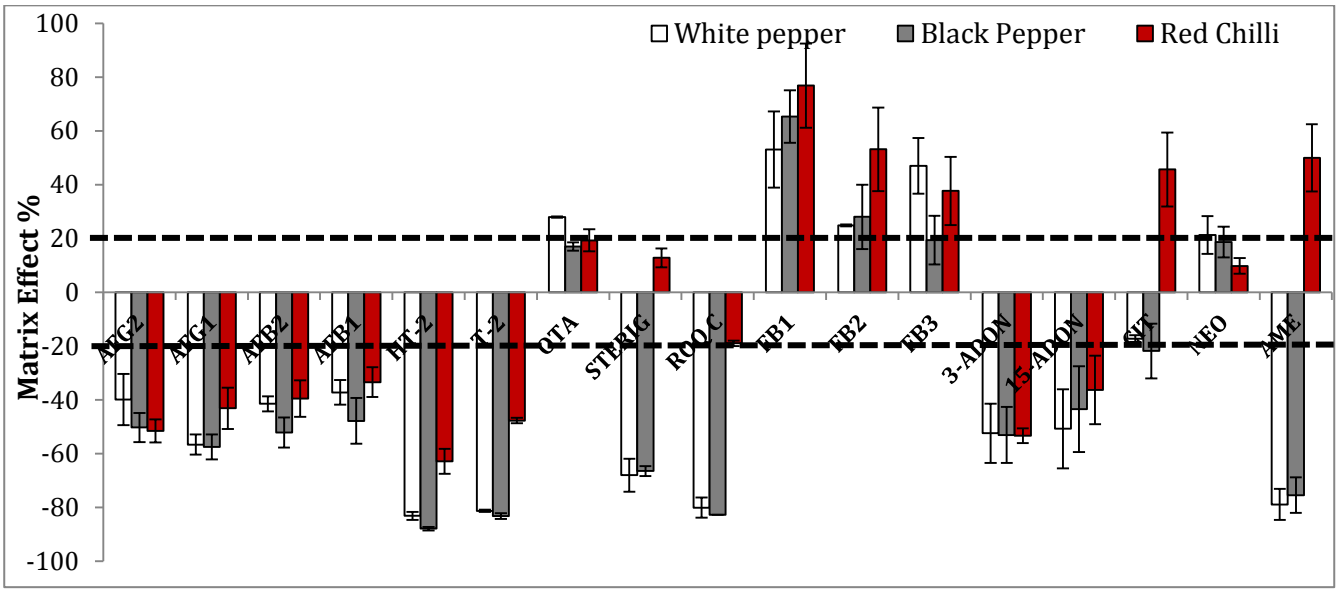

Fig. 2-6. Matrix effects of different spices on the response of each mycotoxin. The concentration range used for the ME evaluation were, AFB1, AFB2, AFG1 and AFG2 (5-150 µg/L), OTA (10-300 µg/L), STERIG (6.25-187.5 µg/L), T-2, HT-2, ROQ C, NEO, 3-ADON, 15-ADON, FB1, FB2, FB3, AME and CIT (25-750 µg/L). A tolerance level of matrix effect is shown between the two dashed lines.

A range in between (-20) to +20% ME or SSE in between 0.8 to 1.2 was considered as tolerable (Frenich et al., 2011). Values outside this range indicate severe ME. It can be seen that, OTA in black pepper and OTA, STERIG, ROQ C and NEO in red chilli are the only analytes close to the tolerable range of ME. Signals for AFs in all the matrices were suppressed by 37 to 68%. A very strong ion suppression effect was observed on T-2, HT-2, STERIG, AME and ROQ C in black and white pepper (65-85%). In the study of Amate et al. (2010), 67% of the compounds had a strong signal suppression effect on black pepper, and it was stated as the most critical matrix. All the three types of fumonisins showed an enhancement effect regardless of the type of spices. Ion enhancement of fumonisins was also reported in beer samples (Tamura et al., 2011) extracted with QuEChERS approach.

Nevertheless, to compensate these significant ME and to improve the linearity, reliability and accuracy of the analytical results MMC curves were used. Moreover, the residual co-extractives were determined gravimetrically, by weighing the dried residue after evaporating the solvent under N_2. The mean (n=5) residual co-extractives obtained after QuEChERS extractions, 8.94±0.99, 10.69±0.99, 9.37±1.59 mg/mL, in white pepper, black pepper and red chilli, respectively, showed that there were no significant differences between different spices. However, the ME was found to be significantly different for some analytes between matrices. It is noticeable with STERIG and AME, on which both peppers had a significant ion suppression (80%) while chilli had 17% and 50% of ion enhancement, respectively. For citrinin, ME in peppers were within the acceptable range, but signal enhancement of 40% was found with chilli matrix. It is conceivable that in addition to the amount of matrix components, the types of matrix components will also have an impact on analyte results.

A selective sample preparation to eliminate the matrix components is rather difficult and may risk significant losses of some trace analytes. Finding appropriate IS in a multi-component analysis is often challenging. A single IS cannot compensate the encountered matrix effects, as it would be different with each analyte on each spice. The deuterated or isotopically labeled standard could have been used for each analyte, but it was avoided considering the cost of multi-toxin analysis. ZAN (IS) was used only for quality control purpose throughout our study; to additionally ensure the constant retention time during the analysis. Moreover, no significant differences were obtained for most of the analytes, when comparing the recoveries calculated using the peak areas and the relative peak areas (analyte peak area divided by the peak area of ZAN at fixed concentration 500 µg/kg)). As an example in red chillies, the recoveries (mean ± SD) of AFG2, AFG1, AFB2 and AFB1 obtained at 40 µg/kg using the relative peak areas were 110±4, 116±11, 102±6, 103±16, respectively. Most of these values are also within the acceptable recovery range of the required performance criteria (EC, 2006a), though ZAN is an inapt IS in this case. Nevertheless, the previous studies state that the ME might not be completely eliminated and it is already an established fact that ESI is more prone to ME than atmospheric pressure chemical ionization (APCI) (Modhave, 2012). Finally, the validation parameters for each spice matrix were determined based on the absolute peak area from the respective MMC curves.

2.3.3. Method validation

Method validation was performed in terms of linearity, repeatability, reproducibility, LODs, LOQs and selectivity for all the three different spice matrices. A cut-off (CO) concentration level for each analyte (a limit to distinguish high and low contamination level) was decided prior to validation by

analyzing some spice samples. For AFB1 and other AFs, a CO concentration of 10 µg/kg was fixed, since contaminations of most of the samples analysed were close to this value. Hence, validation of very low concentrations (less than 0.5 times of CO) was considered unnecessary. For the non-detected ones, rather higher CO (200 µg/kg for all the fumonisins) values were determined based on the judgment on the MLs set on other foods (e.g., Fumonisins in unprocessed maize is 2000 µg/kg and for maize flour it is 1000 µg/kg) (EC, 2006b).

2.3.3.1. Linearity, LOD, LOQ and recovery

MMC curves developed on different blank spice matrices were linear over the working concentration ranges in all of the studied mycotoxins. Residual plots of each mycotoxins were assessed to ensure the linearity of the model. Calibration curves fitted by linear regression showed coefficients of determination (R^2) ranging from 0.9844 to 0.9997 in red chilli, 0.9890 to 0.9988 in black pepper and 0.9903 to 0.9995 in white pepper (Table 2-2). These statistical measures explain how well the future outcomes could be predictable by our model on each of the spice matrices.

The LOD values of the tested mycotoxins ranged from, 1.2 to 73 µg/kg in red chilli, 2.0 to 33 µg/kg in black pepper and 1.3 to 44 µg/kg in white pepper. The LOQs of different spices ranged from 2.3 to 146 µg/kg (Table 2-2). The highest LOQs obtained were for CIT in both black pepper and red chilli and in white pepper it was FB3. The lowest LOQs obtained by this method were for AFs, followed by OTA in all the spice matrices. The LOQs for both of these toxins meet the regulatory limits set by the EC (EC, 2010a & 2010b). Validation for total AFs as a whole may not be very essential, since the LOQs of the highly toxic AFB1 meets the EU ML of 5 µg/kg and a decision on a lot could be made. Therefore, this simple and straightforward method allows the assessment of the compliance of spices with the EU MLs.

Moreover, the LOQs were quite satisfactory with other toxins like fumonisins, when comparing with the regulations in cereals (Van Egmond et al., 2007). The method allows to simultaneously analyze several chemically diverse mycotoxins on a matrix of analytical complexity and achieving too high sensitivity is highly challenging with such simple and cost-effective extraction method. Improving the method sensitivity may be needless, when the method LOQs meets already the MLs of the regulated toxins. Time-consuming and costly SPE clean-ups possibly can improve the method sensitivity, however it may limit the scope of the target analytes.

Table 2-2. Limit of quantification (LOQ) and coefficients of determination (R^2) obtained for black pepper, white pepper and red chilli.

Mycotoxin	Black pepper		White pepper		Red chilli	
	LOQ (µg/kg)	R^2	LOQ (µg/kg)	R^2	LOQ (µg/kg)	R^2
AFG2	4.5	0.9985	2.5	0.9995	3.3	0.9992
AFG1	5.0	0.9973	3.8	0.9989	4.7	0.9976
AFB2	4.0	0.9984	3.7	0.9994	3.0	0.9993
AFB1	4.0	0.9988	3.1	0.9993	2.3	0.9994
T-2	47	0.9935	18	0.9991	20	0.9989
HT-2	42	0.9948	27	0.9978	23	0.9967
FB1	64	0.9969	82	0.9950	64	0.997
FB2	68	0.9965	82	0.9951	64	0.997
FB3	43	0.9986	88	0.9916	80	0.9952
OTA	13	0.9970	12	0.9986	4.2	0.9997
STERIG	8.0	0.9973	16	0.9937	11	0.9946
ROQ C	14	0.9964	4.7	0.9990	17	0.9948
CIT	65	0.9988	84	0.9948	146	0.9844
NEO	32	0.9970	77	0.9903	37	0.996
3-ADON	47	0.9935	40	0.9954	42	0.9948
15-ADON	61	0.9891	76	0.9954	46	0.9937
AME	Not detectable in both peppers				53	0.9979

Regarding LOD and LOQ, there are several possible conceptual methods, each providing a somewhat different definition. Signal- to noise (International Conference on Harmonisation ICH), blank determination, linear regression, limit of blank (LoB) are generally used to determine LOD and LOQs (Vial and Jardy, 1999; Shrivastava and Gupta, 2011). A signal-to-noise ratio (S/N) of 3 is generally accepted for estimating LOD and S/N of 10 is used for estimating LOQ. This method is commonly applied to analytical methods that exhibit baseline noise. The blank determination is applied when the blank analysis gives results with a non-zero standard deviation (SD), however, it is difficult to get a blank yielding non-zero SD. For a linear calibration curve, it is assumed that the instrument response y is linearly related to the standard concentration x for a limited range of concentration. This method can be applied in all cases, and it is most applicable when the analysis method does not involve background noise. It uses a range of low values close to zero for calibration curve. The linear regression method can help to solve the problem of difficulty in obtaining matrix blank for other methods, because calibration curve can be prepared by sample addition method. LoB is estimated by measuring replicates of a blank sample and calculating the mean and the SD (LoB = mean blank + 1.645(SD blank)). LOD is determined by utilising both the measured LoB and test replicates of a sample known to contain a low concentration of analyte (LOD = LoB + 1.645(SD low concentration sample)).

Depending on the definition chosen, the values of LOD and LOQ can vary greatly (by a factor of 5 to 6) for the different methods which make it difficult for comparative purposes. It is therefore essential to have a clearly described procedure for estimating the LOD and LOQ during method validation to allow inter-laboratory comparisons. The signal-to-noise might present about two times values of LOQ as compared to blank determination method. LOQ values obtained by blank determination were comparable to linear regression method (Vial and Jardy, 1999; Shrivastava and Gupta, 2011).

Mean recoveries for all the tested mycotoxins were in the range of 75-117% (Table 2-3), within the acceptable range of required performance criteria (EC, 2006a). According to the Commission Decision 2002/657/EC (EC, 2002), "trueness" means the closeness of agreement between the average value obtained from a large series of test results and an accepted reference value. Trueness can be determined using the certified reference materials (CRM). It is acceptable to assess the trueness of measurements through recovery of additions of known amounts of the analyte(s) to a blank matrix, if no CRMs are available. Therefore, the term apparent recovery was found suitable to use in place of trueness as we used the spiked spice samples. This is also stated as total recovery of a method (Sulyok et al., 2006). The recoveries obtained with our method were comparable with those described in a very recent publication for red chilli and black pepper (Lacina et al., 2012). However, the LOQs reported were higher (4.8 to 120 µg/kg) compared to our method for all the toxins, except AFG1.

2.3.3.2. Intra-day repeatability and inter-day reproducibility

Relative standard deviations (RSD) were calculated under intra-day repeatability (RSDr) and inter-day reproducibility (RSDR) conditions. The results are summarized in Table 2-3. RSDr values were within the acceptable range of <20% for almost all the analytes matching with the performance criteria requirement of the EC (2006a). However, RSDr for OTA was 24% in black pepper. Comparatively, higher RSDr (up to 22-36%) and RSDR (up to 22-41%) values were obtained for ADONs in different spice matrices. RSDR values were high for NEO in both red chilli (23%) and white pepper (29%) and also for FB3 (26%) in red chilli. The higher variability of the ADONs and NEO could be due to the higher polarity of these mycotoxins compared to others as stated earlier. The salt induced partitioning could have hindered its adequate transfer to the MeCN phase. Poor performance criteria of the polar DON was also observed with this extraction, so it was kept out of the methods scope for validation and can only be determined qualitatively.

Table 2-3. Intra-day repeatability (RSDr), inter-day reproducibility (RSDR) expressed as relative standard deviation (%) and apparent recovery (%) obtained for black pepper, white pepper and red chilli at two concentration levels for each mycotoxin.

Mycotoxin	Concentration (µg/kg)	Black pepper			White pepper			Red chilli		
		RSDr (%)	RSDR (%)	Apparent recovery (%)	RSDr (%)	RSDR (%)	Apparent recovery (%)	RSDr (%)	RSDR (%)	Apparent recovery (%)
AFG2	5	14	20	117	12	20	111	18	19	91
	40	9	8	100	4	7	100	10	13	93
AFG1	5	8	15	108	16	11	99	12	20	103
	40	6	4	101	7	6	99	10	8	103
AFB2	5	16	16	84	10	9	109	10	11	78
	40	4	8	100	4	7	99	20	14	102
AFB1	5	6	16	79	14	4	95	5	10	104
	40	7	8	102	4	7	99	20	13	102
T-2	50	20	23	106	10	23	95	12	16	103
	200	9	34	83	8	10	99	13	10	99
HT-2	50	25	17	98	16	12	112	11	17	106
	200	9	8	101	7	7	101	9	10	98
FB1	100	8	22	103	16	14	106	23	20	101
	400	7	31	83	11	7	102	6	8	102
FB2	100	18	15	105	17	18	99	9	11	106
	400	11	9	101	4	4	102	7	6	101
FB3	100	22	15	107	7	18	108	23	26	109
	400	9	7	102	6	15	102	12	14	98
OTA	20	24	18	103	21	18	104	15	13	98
	80	10	8	98	5	9	104	7	7	104
STERIG	12.5	13	21	97	9	15	94	5	16	107
	50	9	6	102	9	7	100	4	4	101
ROQ C	20	12	13	103	8	7	102	11	14	113
	80	8	7	101	7	7	93	8	6	100
CIT	100	7	10	106	11	10	101	6	19	103
	400	5	4	101	10	8	102	1	6	97
NEO	50	15	17	102	15	29	75	20	23	99
	200	16	30	85	8	13	105	12	9	99
3-ADON	50	22	15	110	12	41	111	22	30	91
	200	22	15	102	5	7	100	10	12	102
15-ADON	50	36	33	102	19	22	100	20	13	89
	200	16	19	100	4	6	100	15	15	97
AME	100	Not detectable in both peppers						9	13	101
	200							8	9	99

2.3.3.3. Specificity

The power of discrimination between the analyte and closely related matrix components, known as specificity (EC, 2002) of the proposed method was evaluated by analyzing blank samples. The absence of any chromatographic signal close to the retention time as of the target compounds indicated the absence of any matrix interferences, despite the high complexity of the matrices. Carry-over of the analytes from the previous sample was evaluated by analyzing the mobile phases

after the highest spiked sample. No carry-overs was observed, as there were no peaks detected in the elution zone of the analytes of interest.

2.3.4. Application of the method on real samples

Following the optimization and validation of the analytical method, it was applied on 30 commercially available samples collected from Sri Lanka. For each spice matrix, ten samples were analyzed to determine the contamination levels. The results of the analyses are summarized in Table 2-4.

Table 2-4. Frequency (ratio of positives/number of samples) and mean contamination of positives (µg/kg) of different mycotoxins found in red chilli, black pepper and white pepper samples collected from Sri Lanka (In case only one sample is quantifiable, the value of that sample is presented. If more than one quantifiable samples then the mean is presented).

Type of spice	AFG2	AFB2	AFB1	OTA	STERIG	FB1	FB2	CIT
Red chilli (n=10)[a]	ND[b]	2/10; <LOQ[c]	3/10; <LOQ[d] 6/10; 18[e]	2/10; <LOQ 4/10; 13[d]	4/10; <LOQ	ND	5/10; <LOQ	ND
Black pepper (n=10)[a]	1/10; 5.7	ND	1/10; 11	3/10; < LOQ 1/10; 48	5/10; <LOQ	1/10; 134.5	ND	1/10; <LOQ
White pepper (n=10)[a]	1/10; 2.6	1/10; <LOQ	1/10; 4.9	ND	4/10; 24 4/10; <LOQ	ND	ND	ND

[a]No of samples; [b]Not detected; [c]Limit of quantification; [d] 3 of the 10 samples were <LOQ; [e]mean contamiantion of the 6 positive samples of the 10.

MMC curves were developed for each spice matrix for accurate quantification of mycotoxins. Apart from the selection of two MRM transitions, the relative ion intensity of the real samples was compared with that of the spiked samples as additional selectivity criteria (EC, 2002).

Out of the ten red chilli powders six samples were found to be contaminated with AFB1 in the range of 5.1-35 µg/kg, exceeding the EU ML of 5 µg/kg (EC, 2010a). Moreover, three out of the ten red chilli samples were contaminated simultaneously with four different toxins namely, AFB1, STERIG, OTA and FB2. Earlier 65% of the chilli samples were found to contain more than one toxin from a Spanish study which included AFs, OTA and zearalenone (Santos et al., 2010). OTA was found in four red chillies with a range of 7-27 µg/kg (mean concentration 13.3 µg/kg). Chromatograms of some contaminated spice samples are shown in Fig. 2-7.

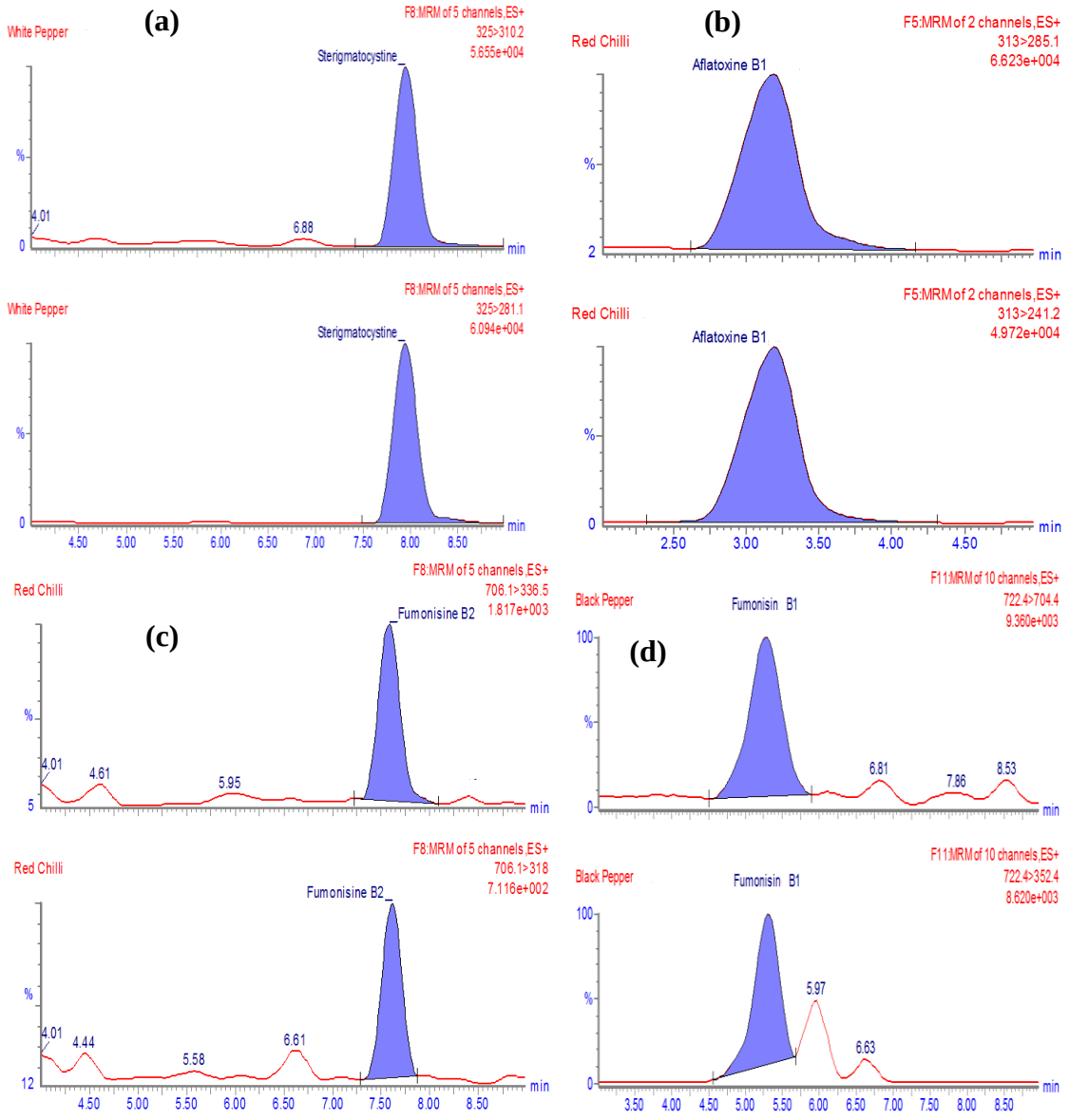

Fig. 2-7. LC-MS/MS chromatograms of some positive spice samples: a) white pepper contaminated with 36 µg/kg STERIG; red chilli with b) 18 µg/kg AFB1 and c) <LOQ FB2 and d) black pepper with 134.5 µg/kg FB1. Quantification and confirmation transitions are shown for all the contaminated samples.

AFBI and AFG2 were detected only in one white pepper sample. 50% of the red chilli samples were positive for FB2. Incidence of FB2 in red chilli was not reported elsewhere till date. Concentrations of AFB1 and OTA found in each of the black pepper sample were above the EU ML (EC, 2010a & 2010b). Considering, the Indian black pepper samples 54% of them were positive for OTA in the range of 10-51 µg/kg according to Thirumala-Devi et al. (2001). CIT was also detected in one black pepper sample (<LOQ) in our study. Meanwhile, highest STERIG contamination was found in white pepper, ranging from 15 to 36 µg/kg. However, compared to the black pepper, overall AFs and/or OTA contamination in white pepper is much lower. Despite the reported inhibitory action of the pepper pungent principle piperine on fungal growth and mycotoxin production (Madyastha and

Bhat, 1984), contamination in pepper seems inevitable. It should be noted that many previous studies failed to detect several other mycotoxins except AFs and OTA in different spices (Aydin et al., 2007; Fazekas et al., 2005; Jalili et al., 2010; Amate et al., 2007). This study revealed that apart from these 'classical' mycotoxins associated with spices other toxicologically relevant mycotoxins were also found to be present.

2.4. CONCLUSIONS

A simple quantitative method based on a QuEChERS extraction approach, for simultaneous determination of multiple mycotoxins in three spices (chilli, black and white pepper) using HPLC-MS/MS was developed and successfully validated. The scope of the QuEChERS technique was further extended by its application on the extremely complex spices for mycotoxin analysis. MS/MS detection increased the confidence of analyte identification in spices. Significant matrix effect was successfully compensated using matrix matched calibration curves. The method LOQ meets the maximum levels of the two regulated toxins aflatoxins and ochratoxin A in spices hence, it can be used for the purpose of enforcement of the proposed EU MLs. It is an effective tool for quantitative screening of diverse mycotoxins in spices and it can be useful for exposure assessment studies. This time saving and cost efficient method is also very flexible and new compounds like pesticide residues can be added easily. The clean chromatograms obtained on real sample matrices indicate the reliability of the method for confirmatory purposes.

The developed analytical method was subsequently applied to screen the presence of multiple mycotoxins in pepper and chilli samples collected from Sri Lanka and Belgium. The contamination results of pepper and chilli are presented in **Chapter 3 and 4**, respectively.

CHAPTER 3

MYCOLOGICAL QUALITY AND MYCOTOXIN CONTAMINATION OF SRI LANKAN PEPPERS (*Piper nigrum* L.)

CHAPTER 3: MYCOLOGICAL QUALITY AND MYCOTOXIN CONTAMINATION OF SRI LANKAN PEPPERS (*Piper nigrum* L.) AND SUBSEQUENT EXPOSURE ASSESSMENT

Summary

The aim of the study was to characterize the toxigenic moulds and to screen different mycotoxins in peppers (*Piper nigrum* L.) of Sri Lankan origin. *Aspergillus flavus, Aspergillus parasiticus, Aspergillus niger* and *Penicillium* spp. were found to be the most dominant fungi. Characterization of the moulds was carried out in *Aspergillus flavus* and *parasiticus* agar (AFPA) and malt extract agar (MEA) in 77 black pepper and 11 white pepper samples collected during 2011-2012. In total, 73% of the black pepper and 64% of the white pepper samples were contaminated with *Aspergillus flavus* and/or *Aspergillus parasiticus (*AfAp*).* A black pepper sample with water activity (a_w) 0.70 recorded the highest count of AfAp ($4.3x10^4$ CFU/g). Moreover, 75% of the black pepper samples exceeded the safe a_w limit (0.65) set by the European Spice Association (ESA). The frequency of occurrence of *A. niger* in black pepper was 62% with counts up to $1.3x10^3$ CFU/g. *Penicillium* spp. were found in 61% and 55% of the black pepper and white pepper samples, respectively. In black pepper 94% of the samples had a *Penicillium* contamination below 10^3 CFU/g. Other *Aspergillus* spp, found in peppers included, *Aspergillus terrus, Aspergillus tamarii, Aspergillus candidus, Aspergillus penicilloides, Aspergillus sydowii* and *Aspergillus fumigatus*. Mould counts in black pepper (10^2-10^4 CFU/g) were significantly higher than that of white pepper (<10^2 CFU/g). Apart from the occurrence of "classical mycotoxins" of spices, aflatoxins (<LOQ-17 µg/kg) and ochratoxin A (<LOQ-79 µg/kg), other toxins including fumonisin B1 (<LOQ-135 µg/kg), sterigmatocystin (<LOQ-49 µg/kg) and citrinin (<LOQ-112 µg/kg) were detected in peppers. In total, 63% of the black pepper samples were contaminated with at least one mycotoxin. Mycotoxin contamination in white pepper was significantly less compared to black pepper. The exposure to aflatoxins and ochratoxin A by consuming pepper remains harmless considering the existing pepper dietary intake data of the Sri Lankan population.

Keywords: *Black Pepper, Sri Lanka, Aspergillus, Toxigenic moulds, Mycotoxins, Exposure assessment*

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3.1. INTRODUCTION

Sri Lanka is the seventh largest producer of pepper in the world (24,950 MT), following Vietnam, Indonesia, India, Brazil, China and Malaysia (FAO, 2012). Sri Lankan pepper is exported to USA, UK and many other EU countries. Sri Lankan pepper fetches a premium price in the world trade because of its high content of the pungent principle piperine (7-15%) (Jansz et al., 1983). Pepper ranks first among spices production in Sri Lanka in terms of value and it is on the top ten in terms of spice world trade (FAO, 2011; Chapter 1).

Fungi that belong to the genera *Aspergillus*, *Fusarium* and *Penicillium* pose serious mycotoxicological risks in a wide range of food products because they can produce several mycotoxins causing health problems in animals and humans. Spices are not exceptional in this regard as they may be exposed to a wide range of microbial contamination from farm-to-fork. The traditional method of drying pepper in open air under the sun, is still common practice in Sri Lanka, which potentially exposes them to the risk of contamination. There are studies on mould contamination in pepper originating from Egypt (Abou Donia, 2008), Brazil (Gatti et al., 2003; Freire et al., 2000), Saudi Arabia (Bokari, 2007; Hashem and Alamri, 2010), Bahrain (Mandeel, 2005) and India (Geeta and Reddy, 1990).

The toxigenic species of *Aspergillus* and *Penicillium* represent a higher risk for food and feed products in storage (Pitt and Hocking, 2009), hence they are generally referred as "storage fungi". Growth of *Aspergillus flavus* and subsequent production of aflatoxins (AFs) occurs under conditions of high relative humidity (>85%), high temperature (>25°C) and insect or rodent activity (CAST, 2003). AFs are produced by certain strains of *A. flavus* and *A. parasiticus* via a biosynthesis route including STERIG (AFB1, AFG1) or dihydrosterigmatocystin (AFB2, AFG2) as the immediate precursors (Sweeney and Dobson, 1999; Cleveland et al., 2009). The natural occurrence of mycotoxins in herbs and spices has increasing interest because of the wide spread use of these substances in the world.

On a global scale, contamination by mycotoxins in peppers was previously reported in Egypt (El-Kady et al., 1995; Aziz et al., 1998), Portugal (Martins et al., 2001), Italy (Romagnoli et al., 2006), Malaysia (Jalili et al., 2010) and Morocco (Zinedine et al., 2006). However, the type and level of different mycotoxins vary in each country as well as in different regions of a country depending mainly on the environmental conditions where it has been cultivated and the storage/transport conditions.

Apart from the two regulated mycotoxins in the EU, aflatoxins and OTA, determination of other types of toxins has not been carried out, since a method for the simultaneous analysis of multiple mycotoxins in spices was lacking for long time. There were no studies so far on characterizing the mycotoxigenic moulds and/or the co-occurring mycotoxin contamination in Sri Lankan peppers, despite it being an economically significant spice crop of the island. The present investigation aims in assessing the intensity and frequency of the mycotoxigenic species with special emphasis on highly toxic aflatoxigenic spp., and the occurrence of different mycotoxins in peppers grown and processed in Sri Lanka. Furthermore, a deterministic exposure assessment was carried out on consumption of peppers and comparison was made with several other countries, since Sri Lankan pepper is exported worldwide.

3.2. MATERIALS AND METHODOLOGY

3.2.1. Chemicals and reagents

Malt Extract Agar (MEA) (malt extract 30 g/L, mycological peptone 5 g/L and agar 15 g/L), selective identification medium *Aspergillus flavus* and *parasiticus* agar (AFPA) (peptone 10 g/L, yeast extract 20 g/L, ferric ammonium citrate 0.5 g/L, dicloran 0.002 g/L and agar 15 g/L) and chloramphenicol supplement (1 vial per 500 mL medium) were supplied by Oxoid (Hampshire, England). Formic acid ULC-MS grade (99%) was supplied by Bio Solve BV (Dieuze, France). Other chemicals and reagents used for LC-MS and for sample preparation were of analytical grade, same as described in **Chapter 2 (section 2.2.1).**

3.2.2. Mycotoxin standards

Mycotoxin reference standards used were the same and the working standard solutions were prepared as described in **Chapter 2 (section 2.2.2).**

3.2.3. Samples and data collection

The spice samples (~200-500 g) of black pepper (n=82) and white pepper (n=11) were collected from Sri Lanka (farm-to-fork) during 2011-2012. Number of black pepper samples along the supply chain included, farmers (n=11), processors (n=23), local traders (n=29) and exporters (n=14), while 5 were un-categorised. Most of the black pepper samples were collected from Kandy and Matale district (n=42), where pepper is generally grown and also from Jaffna (n=19) and Anuradhapura (n=16) district. Sampling regions and their corresponding agro-ecological zones are shown in Fig. 3-1. Regarding white pepper, eight samples were from the local traders of Kandy/Matale, two from Anuradhapura and one from Jaffna. Different forms of black pepper included whole pepper (n=49), crushed pepper (n=6), pepper powder (n=17) and light berries (n=5). The samples collected in open markets were packed air-tight in low density poly ethylene (LDPE) and transferred to Belgium. All

the pre-packaged samples were stored at room temperature and the samples collected from open markets were stored at 4°C until mould isolation and multi-mycotoxin analysis. For comparison reasons, some black pepper (n=20) and white pepper (n=7) samples were additionally collected from different markets in Belgium, mainly exotic shops. All the pepper samples were finely ground (M20 IKA®-WERKE; Staufen, Germany) prior to both mould isolation and mycotoxin analysis. Farmers, local traders, processors, exporters and experts from the Department of Export Agriculture, Sri Lanka were interviewed in order to collect the data on pre- and post-harvest practices on pepper.

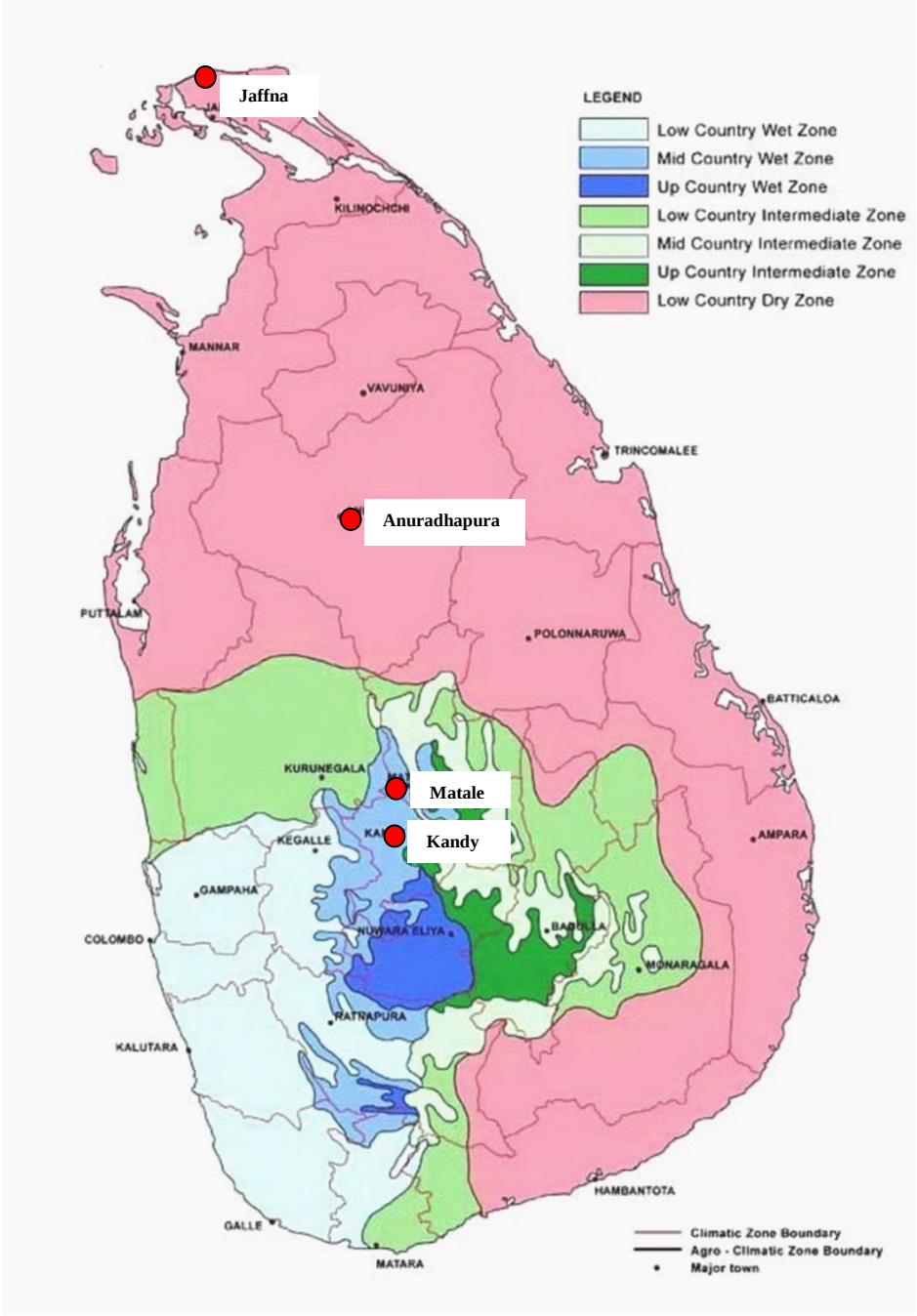

Fig. 3-1. Agro-ecological zones of Sri Lanka (Source: Department of Agriculture, Sri Lanka) showing sampling regions marked with red circles (Top to bottom: Jaffna, Anuradhapura, Matale and Kandy).

3.2.4. Isolation and identification of moulds

Moulds were isolated from 77 black pepper samples (except light berries (immature) which are often used for oleoresin production, normally harvested at 5-6 months of maturity) and 11 white pepper samples. Prior to the mould isolation, moisture measurements of peppers were taken using the oven dry method at 105°C until their weights remained constant. Water activity (a_w) was measured using a a_w meter (Novasina LabMaster, Lachen SZ, Switzerland). For pH measurement, a spice suspension was prepared at 1:10 ratio (spice:water) using demineralized water. After 24 hours of shaking on a planar shaker (Heidolph Unimax 2010, Schwabach, Germany), the pH was measured using a ThermoOrion pH meter. Mould

For mould isolation, 10 g of the ground black pepper sample was initially diluted with 90 g of 1% sterile peptone water (8.5 g NaCl and 1 g bacteriological peptone per litre of demineralized water) in a stomacher bag and extraction was performed with stomacher (Seward stomacher 400, Zolder, Belgium), at normal speed for 30 min. Then, 5 mL of the spice extract was spread plated on 15 Petri plates of each MEA and AFPA media supplemented with chloramphenicol in ethanol. All the visually moulded and non-moulded pepper samples were screened for different moulds. The plates were incubated at 30°C. Regular observation on the mould growth was performed in each plate and on each media visually and microscopically. Mould identification and plate counts were carried out after 7 days of incubation. Plates were stored in refrigeration at 4°C for further identification and counting of the moulds.

One of our main objectives of this study was to characterize the toxicologically significant moulds in black peppers. A selective differential medium AFPA was used for the counting of *Aspergillus flavus* and *Aspergillus parasiticus* as recommended. Moreover, our interest was also to characterize the other moulds (other *Aspergillus, Penicillium, Fusarium* spp. etc) that are potentially toxic. For this purpose MEA was used in our study. Though DG-18 is generally recommended for enumeration and isolation of yeasts and moulds from dried and semi-dried foods, it was not used in our study. DG-18 generally allows the growth of all the moulds and yeasts (xerophillic). The toxicologically insignificant highly xerophillic ascomycete filamentous fungus, *Xeromyces bisporus* (minimum a_w 0.61) or the highly xerophillic yeast *Zygosaccharomyces rouxii* (minimum a_w 0.62) were not given much importance in this study, though they were also known to be present in spices. Secondary metabolites were not detected experimentally, since gene clusters encoding for secondary metabolites were noticeably absent in these species (Leong et al., 2014). So they are not toxicologically relevant. It has been also shown that, at a_w<0.80, *X. bisporus* was competitive because it grew faster than the other species (Leong et al., 2011). Moreover, the xerotolerant species, *A. flavus* and *Penicillium roqueforti* were shown to be dominant over *X. bisporus* only at

0.94 a_w and at lower a_w generally *xerophilic* species over grow. Diluting the extract too high in order to ease the mould counting might lead to the underestimation of the important toxic moulds. Therefore, to facilitate the counting of the toxicologically significant mould colonies, MEA was used in this study (total moulds and yeasts count was not the target). Nevertheless, moulds other than *Aspergillus* and *Penicillium* spp. that were able to grow in MEA were also counted and reported collectively as other moulds.

Identification of moulds at genus and/or species level was performed directly on agar plates using the stereo microscope (Olympus SZX16, Tokyo, Japan). Upon necessary, further isolation and purification was performed by preparing pure cultures on fresh MEA plates. Moulds were characterized under microscope using the criteria or appropriate keys described by Bennet (2010); Pitt and Hocking (2009); Samson et al. (2004) and Klich (2006), on their colony morphology and spore characteristics. Inverse microscope (Olympus IX81, Tokyo, Japan) was used to check the colonies in detail after colourizing with aniline blue droplet. Appropriate dilutions were made when the colonies were uncountable. Number of colonies of each species or at genus level were expressed as colony forming units per gram (CFU/g) of the sample.

3.2.5. Extraction of samples for mycotoxins analysis

Samples were extracted using the QuEChERS based approach as described in **Chapter 2 (section 2.2.4).** The instrumentation and the conditions were the same as described in **Chapter 2 (section 2.2.5).**

3.2.6. Statistical analysis

The Kolmogorov-Smirnov (K-S) and/or Shapiro Wilk test together with the corresponding Q-Q plots were used to determine the normality of the data distribution. Due to the non-normal distribution of the mycotoxins and mould contamination data, the non-parametric Kruskal-Wallis one way ANOVA was used to assess the significance and post-hoc pairwise multiple comparisons were made to identify the differences among the variables upon significant results. Box-and-whisker plots were also used as well to identify differences between the variables by assessing the medians and/or interquartile range (IQR) of mould and mycotoxin contamination levels in peppers. Statistical analyses were performed on the log values of the mould counts. Spearman correlation coefficients were used to identify the correlations among mycotoxins and moulds between different sample groups. The statistical analyses were carried out using the SPSS statistical package (IBM®, Version 21). Level of significance was 0.05 unless otherwise specified.

3.3. RESULTS AND DISCUSSION

3.3.1. Moisture content (MC), water activity (a_w) and pH of peppers

The results of moisture content and a_w measurements of the black pepper samples are shown in Table 3-1. Mean percentage of the MC and a_w of black pepper (n=52) were 13.7±1.7 (mean±SD) and 0.68±0.05, while in white pepper they were 14.4±0.02 and 0.68±0.07, respectively. American Spice Trade Association (ASTA) and European Spice Association (ESA, 2011) state that both MC and a_w are necessary moisture measurements to ensure food safety. Only 23% of the black pepper and 36% of the white pepper samples were in accordance with the MC of 12% required by ESA. ASTA specifies the minimum safe level a_w as 0.75, while a more stringent a_w is set by ESA which is 0.65. Considering these specifications, 17% and 75 % of the black pepper samples exceeded the required a_w limits of ASTA and ESA, respectively. All moisture levels of the black pepper samples exceeded the level of 10% which is the upper limit set by spices board of India (2009).

Table 3-1. Moisture content and water activity of black pepper samples grouped into different categories (number of samples analysed for each measurement are given within brackets).

Main groups	Sub-groups	Moisture content (% wwb) (mean±SD)	Water activity (mean±SD)
Form of pepper	Crushed pepper	14.4±1.67 (3)	0.64±0.03 (5)
	Pepper powder	12.8±0.90 (11)	0.67±0.05 (17)
	Whole pepper corns	12.2±1.20 (30)	0.69±0.06 (49)
	Light berries	14.0±1.11 (3)	0.69±0.02 (5)
Sampling region	Jaffna	13.6±1.52 (8)	0.66±0.04 (19)
	Kandy-Matale	13.7±1.96 (33)	0.70±0.06 (41)
	Anuradhapura	14.0±0.71 (8)	0.67±0.04 (16)
Production line	Farmer	15.0±2.80 (6)	0.73±0.06 (11)
	Processor	12.8±1.47 (14)	0.67±0.05 (23)
	Local trader	13.5±1.33 (16)	0.66±0.03 (28)
	Exporter	14.4±1.40 (13)	0.71±0.05 (14)
Grades	Grade 1	14.4±1.46 (12)	0.71±0.05 (13)
	Grade 2	12.8±1.53 (19)	0.65±0.03 (31)
	Grade 3	13.9±0.70 (8)	0.68±0.05 (16)
	Ungraded	15.0±2.80 (6)	0.73±0.06 (11)
Packaging	Packaged	12.9±1.41 (21)	0.66±0.04 (36)
	Unpackaged	14.3±1.75 (28)	0.70±0.05 (41)

According to Sri Lankan Standards (SLS, 2008), the MC should be 12% for grade 1 and 14% for all the other pepper grades. In total, 58% of the black pepper samples and 27% of the white pepper samples, exceeded the MC limit of 14%. The International Pepper Community (IPC) fixed a standard MC in between 12-15% based on the type and grade of pepper. None of the samples were less than 0.60 a_w, the minimum limit to prevent microbial proliferation (Pitt and Hocking, 2009). These results indicate that the xerophilic moulds could sufficiently grow in these "dry" spices. Strict

control on the safe a_w levels throughout the production steps should be a pre-requisite to ensure longer shelf life and prevention of mould growth and/or mycotoxin production of these costly spices. Furthermore, pH was checked for 15 black pepper and 3 white pepper samples. For black and white pepper, the pH was 6.9±0.6 and 7.0±0.3 (mean±SD), respectively. It has been reported that pH has little or no influence on the growth of moulds (Samson et al., 2004).

3.3.2. Mould contamination in peppers

The results show that *A. flavus* and/or *A. parasiticus, Aspergillus niger* and *Penicillium* spp. were found to be the most dominant toxigenic fungi present in peppers (Table 3-2). According to ASTA (2011), common moulds found in spices and herbs include, *A. flavus, A. niger, A. fumigatus, A. glaucus, A. tamarii, A. terreus, A. versicolor, Absidia* spp., *Mucor* spp., *Penicillium* spp., and *Rhizopus* spp., in the range of 10^2 to 10^6 CFU/g. Of all the spices, black pepper typically had the highest aerobic plate counts, (>10^6 CFU/g) which included both the bacterial and fungal counts.

Table 3-2. Descriptive statistics and frequency on mould contamination of peppers in Ln CFU/g (data transformed using natural logarithm).

A. Black Pepper

Descriptive statistics (n=77)	AFPA	MEA (Ln CFU/g)				
	AfAp[a]	*A. niger*	other *Aspergilli*	*Penicillium*	other noulds	Total moulds
Average	3.42	2.68	5.22	2.36	4.52	6.54
Maximum	10.67	7.17	11.10	9.10	12.67	10.95
Median	3.23	2.80	5.55	2.30	4.63	7.02
Stdev	2.80	2.49	2.61	2.50	2.36	1.97
Inter Quartile Range (IQR)	5.75	4.75	3.54	3.92	2.35	1.99
No. contaminated samples	56	48	69	47	72	74
Frequency of occurrence (%)	73	62	90	61	94	96

B. White Pepper

Descriptive statistics (n=11)	AFPA	MEA (Ln CFU/g)				
	AfAp[a]	*A. niger*	other *Aspergilli*	*Penicillium*	other noulds	Total moulds
Average	1.67	0.94	4.02	2.55	3.89	5.83
Maximum	4.43	4.09	8.67	7.79	8.43	9.26
Median	1.39	0.00	4.06	0.69	4.28	6.15
Stdev	1.57	1.52	2.94	2.94	2.63	1.94
Inter Quartile Range	2.90	1.70	4.66	4.83	0.23	1.04
No. contaminated samples	7	4	9	6	9	11
Frequency of occurrence (%)	64	36	82	55	82	100

[a]*Aspergillus flavus* and/or *Aspergillus parasiticus*

AFPA base which was recommended for the detection and enumeration of the two species, *A. flavus* and *A. parasiticus*, was used in our study. Since both of these moulds form intense

yellow/orange pigmentation underside (Fig. 3-2A and conidiophores in Fig 3-2B) and express very similar morphology they were counted together in all the samples. According to Samson et al. (2004), the single morphological character to distinct these species is the presence of roughened conidia wall texture in *A. parasiticus*. Additionally, mycotoxin analysis was performed in pure cultures prepared in MEA (incubated for 14 days at 30°C) to distinguish the strains of *A. flavus* and *A. parasiticus* since, our aim was also to identify the toxigenic strains for a future study. In total, 38 strains were found to be toxigenic and 29 of them produced aflatoxin B toxins, while only 9 strains produced both B and G toxins. According to Klich (2007), *A. flavus* usually produces B toxins almost all the *A. parasiticus* isolates produce both B and G toxins. Hence, combination of characters were found to be necessary to identify these related fungal species.

Apart from these two *Aspergillus* spp., *A. ochraceus* and *A. niger* were also found to grow in the AFPA medium. However, *A. ochraceus* was not frequently found in our samples. Descriptive statistics of all the identified moulds for black and white pepper are shown in Table 3-2. A very high variability among the counts was observed hence, the data was transformed (natural logarithm/Ln CFU/g) for better comparison. Median and inter quartile range (IQR) could also be used for comparison. In total, 73% of the black pepper and 64% of the white pepper samples were contaminated with AfAp (Table 3-2A). However, the amount of contamination in black pepper (10^2-10^4 CFU/g) was significantly higher (P=0.015) than that of white pepper which had a maximum contamination of only 86 CFU/g. A black pepper sample with heavy mould contamination is shown in Fig. 3-2C. The frequency of occurrence of AfAp in Sri lankan pepper was higher than the frequencies found by Gatti et al. (2003) and Freire et al. (2000) from Brazilian samples and by Hashem and Alamri (2010) from Saudi Arabia. Similar frequencies were reported from Bahrain (Mandeel, 2005).

Furthermore, Bokhari (2007) reported that all the black pepper samples were contaminated with *A. flavus*. In our study, less contamination of moulds in white pepper could be due to the absence of the fleshy mesocarp and epicarp as it is removed during the production process, leaving the thick-walled endocarp of the white pepper to act as a physical barrier to fungal proliferation (Madhyastha and Bhat, 1984). On the other hand, the cracked wrinkled surface of black pepper may provide a conducive environment for mould growth. According to scanning electron microscopic studies, *A. flavus* are colonized more on the ridges of pepper corns (Seenappa and Kempton, 1980).

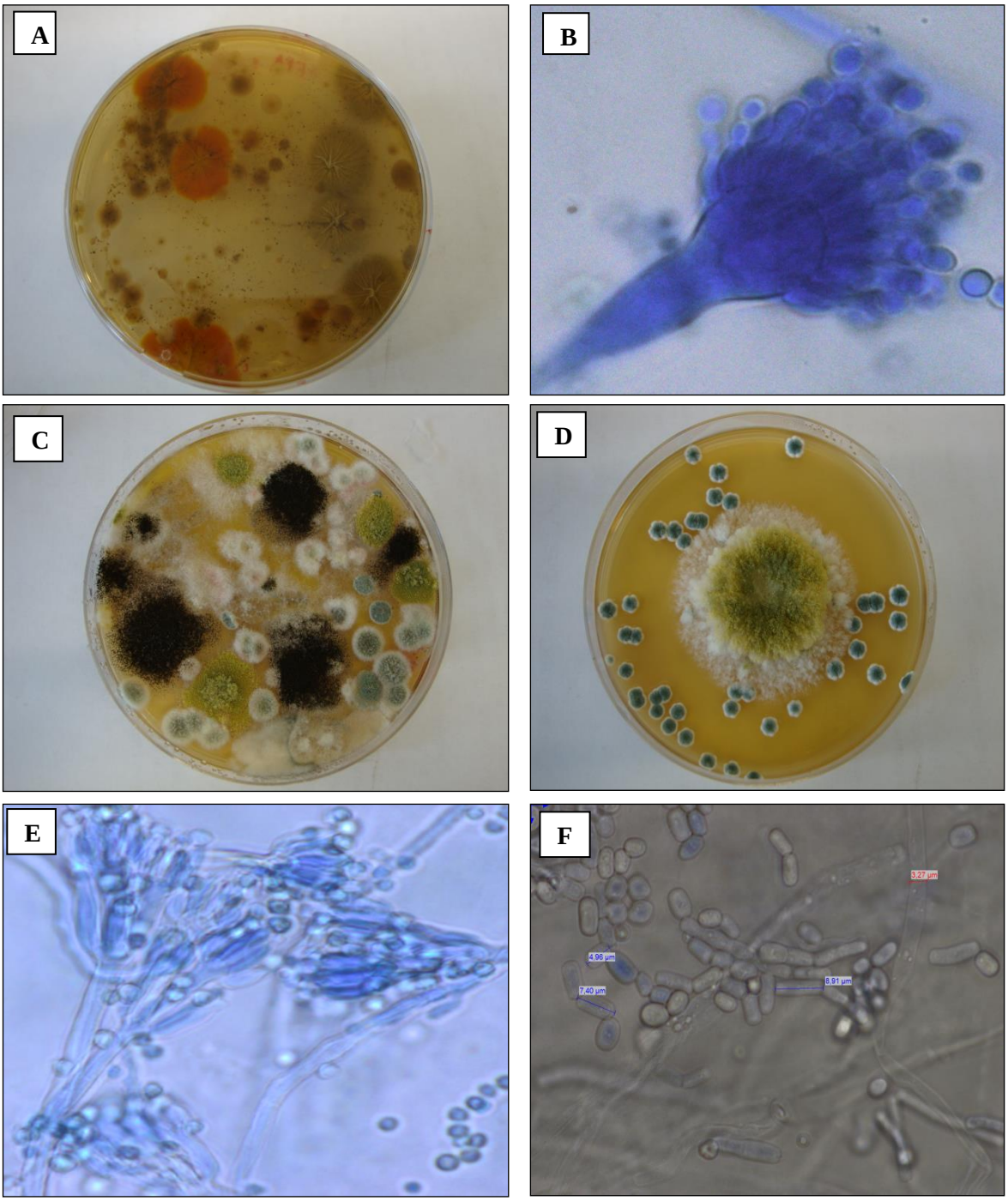

Fig. 3-2. A) *Aspergillus flavus* and/or *Aspergillus parasiticus (AfAp)* isolated from black pepper in AFPA medium showing the orange reverse colouration, B) Conidiophore (x100) of an *Aspergillus* spp. stained with aniline blue, C) a heavily contaminated black pepper with different toxigenic moulds isolated in MEA, D) *Penicillium* spp. and *A. flavus* colony (center) isolated from white pepper in MEA, E) Conidiophore (x100) of a *Penicillium* spp. stained with aniline blue and F) Microscopic image of *Geotrichum candidum* isolated from black pepper.

A black pepper sample with a_w 0.70 recorded the maximum count of $4.3x10^4$ CFU/g AfAp. However, it may not be appropriate to relate this a_w to the high mould count found; mould contamination could have occurred earlier when a_w was high enough for their growth in the field or during drying. Pitt and Hocking (2009) stated 0.79 as the minimum a_w for the growth of *A. flavus*.

Contamination in the scale of 10^4 CFU/g was also reported from India (Geeta and Reddy, 1990). AfAp contamination levels between 10^2-10^3 CFU/g were found in 36% of the Sri Lankan black pepper samples while total mould counts of more than 10^3 CFU/g were found in 52% of the black pepper samples. Only 10% of the black pepper samples were free from *Aspergillus* spp., while only 3% of them were free from mould contamination.

Moreover, *A. niger*, an OTA producing species was very frequently found in both peppers. The frequency of occurrence of *A. niger* in black pepper was 62% with counts up to $1.3x10^3$ CFU/g. Frequency and the level of contamination of *A. niger* in black pepper was significantly higher compared to white pepper (Table 3-2). According to Bokhari (2007), 80% of the black pepper samples were contaminated with this mould with a mean count of $4x10^2$ CFU/g which is similar to our study. Some other studies indicate rather lower frequency of *A. niger* contamination in pepper (Freire et al., 2000; Gatti et al., 2003; Hashem and Alamri., 2010; Mandeel, 2005). In white pepper, 4 out of the 11 samples were contaminated with *A. niger* but with very low levels (maximum 60 CFU/g) (Table 3-2B). A very low frequency of occurrence (4.5%) of *A. niger* in Brazilian white pepper was reported (Freire et al., 2000), however, it has been stated as the second most dominant species next to *A. flavus* in spices.

Other *Aspergillus* spp. found in the pepper samples included *Aspergilus terrus*, *Aspergillus tamarii*, *Aspergillus candidus*, *Aspergillus penicilloides*, *Aspergillus sydowii* and *A. fumigatus*. Since they do not possess high toxicological significance like *A. flavus* or *A. parasiticus*, they all were counted together as other *Aspergilli* (Table 3-2). It should be mentioned that morphological characterization is insufficient to distinguish many *Aspergillus* species, due to the interspecific similarities and intraspecific variability (Rodrigues et al., 2009). Frequency of occurrence of other *Aspergillus* spp. in black and white pepper was 90 and 82% (Table 3-2), respectively.

Penicillum spp. were also frequently found in Sri Lankan peppers. They were found in 61 and 55% of the black pepper and white pepper samples (Table 3-2), respectively. Similar mean contamination levels (10^2 CFU/g) were found in both peppers (Table 3-2). In black pepper 94% of the samples had a contamination below 10^3 CFU/g. Occurrence of *Penicillium* spp. in Sri Lankan pepper samples was higher than reported in Egypt (20%), but lower than from Saudi Arabia (80%). *Penicillium citrinum*, which is known to produce the nephrotoxin CIT and *P. verrucosum* and *P. chrysogenum* which produce OTA were isolated from black pepper (Bokhari, 2007; Mandeel, 2005). In our study, *Penicillium* was identified only at genus level, hence, the mycotoxigenic potentiality could not be assessed. Furthermore, the toxicity of the *Penicillium* mycotoxins is low in comparison to AFs, hence aflatoxigenic moulds were given priority in this study. *A. flavus* and

Penicillium spp. isolated from white pepper are shown in Fig. 3-2D and conidiophores of *Penicillium* spp. in Fig 3-2E.

Other moulds identified in some pepper samples were zygomycota like *Mucor* and *Rhizopus*, *Trichoderma* and *Scopulariopsis*. The incidence of these fungi in pepper was previously reported also by Freire et al. (2000). Furthermore, *Geotrichum candidum* (Fig. 3-2F) which is a common spoilage mould on fruits and found in soil and decaying organic matter (Samson et al., 2004), was identified in 25% of the pepper samples. Remarkably, one black pepper (a_w 0.70) sample which counted $2.3x10^6$ CFU/g of *G. candidum* also had the highest count for AfAp ($4.3x10^4$ CFU/g). As stated earlier, mould contamination could have occurred earlier in the field or during drying and probably surviving at the low a_w reported. It is indeed hard to predict when the actual mould contamination occurred in both type of peppers using our data.

In Sri Lanka, pre-packaged peppers are generally found in supermarkets at rather high prices. Peppers sold in groceries are usually exposed to air and dust and are less expensive. Strangely, significantly lower counts for AfAp ($p=0.004$) and other *Aspergillus* spp. ($p=0.005$) were found with open market samples compared to the packaged ones, regardless of the higher a_w recorded in open market samples. Moreover, no significant differences have been observed with *Penicillium* spp., between unpackaged and packaged samples. Regardless of the high piperine amount in Sri Lankan pepper (Jansz et al., 1983), mould counts were still found in the range of 10^2-10^3 CFU/g. The amount of pepper anti-microbial compounds present may vary with different parameters like climate, cultivar, nutrient availability and drought. Therefore, the amount and type of moulds present on a particular spice could be a multifaceted effect of the matrix compounds, a_w and other environmental parameters.

3.3.2.1. Comparison of mould contamination between different production steps, forms, regions and grades of pepper

Mould contamination was further compared between production steps, different forms, sampling regions and grades of black pepper using box and whisker plots (Fig. 3-3). The level of contamination of AfAp was found to be significantly different ($P=0.003$) among the different steps of the supply chain namely, farmers, processors, local traders and exporters. Moreover, a frequency of occurrence in between 64 to 78% was found among different steps (Fig. 3-4). The counts of AfAp and other *Aspergillus* of the export samples were significantly lower compared to the local traders (Fig. 3-3A). Higher incidence in some of the production steps could be due to improper drying, open market exposure, poor storage etc. Generally black pepper samples from different

farmers are mixed together by the processors and brought to markets. Hence, higher variation of the mould contamination in the supply chain is apparent.

Comparing the different forms of pepper, there were no significant differences on mould counts (Fig. 3-3B), though it is feasible that in crushed pepper and pepper powder, the moulds may be exposed more to the pepper anti-microbial compounds than in the whole corns. Generally, black pepper contains 2-8% of piperine and 2-4% of piperine oil restricted to oleoresin cells and oil cells, respectively (Madhyastha and Bhat, 1984). *In vitro* studies show that 0.1% piperine or 0.01% pepper oil reduces the growth of *A. parasiticus* by 50%. Furthermore, 98% reduction in aflatoxin production has been observed with 1% of piperine (Madhyastha and Bhat, 1984). The effect of these compounds on fungal growth on whole peppercorns can be minimal compared to the powdered or the crushed ones. Based on our occurrence results it is possible to consider that there may be no influence of these compounds in mould growth. Furthermore, no significant differences on a_w were found in between different pepper forms (Table 3-1).

Moreover, different regions were compared on the incidence of mycotoxigenic species (Fig. 3-3C). It can be seen that the samples from Jaffna had significantly higher ($p<0.05$) counts compared to other regions for AfAp, *A. niger*, other *Aspergillus* (Fig. 3-3C) and total mould counts, with frequencies ranging from 48 to 95% (Fig. 3-4C). However, no significant differences in a_w have been found (Table 3-1) between the samples collected from the two low country dry zones, Jaffna and Anuradhapura, which have a mean annual rainfall of <1750 mm and temperature between 26 and 34°C (Department of Agriculture, Sri Lanka, 2013). It could be that somewhere in the supply chain, the a_w was high enough for mould growth in Jaffna samples and later exposure to the dusty environment in the open markets could lead to its higher mould count. The temperature prevailing in this region is highly conducive for AfAp growth. Optimum temperature for *A. flavus* growth is 30°C, while it can grow from 10-48°C, and optimum temperature for aflatoxin production is 25-30°C (Pitt and Hocking, 2009). Kandy and Matale both belong to the mid country wet zone (annual rainfall >2500 mm; humidity 55-85% and mean temperature range 18-33°C), where pepper is extensively grown.

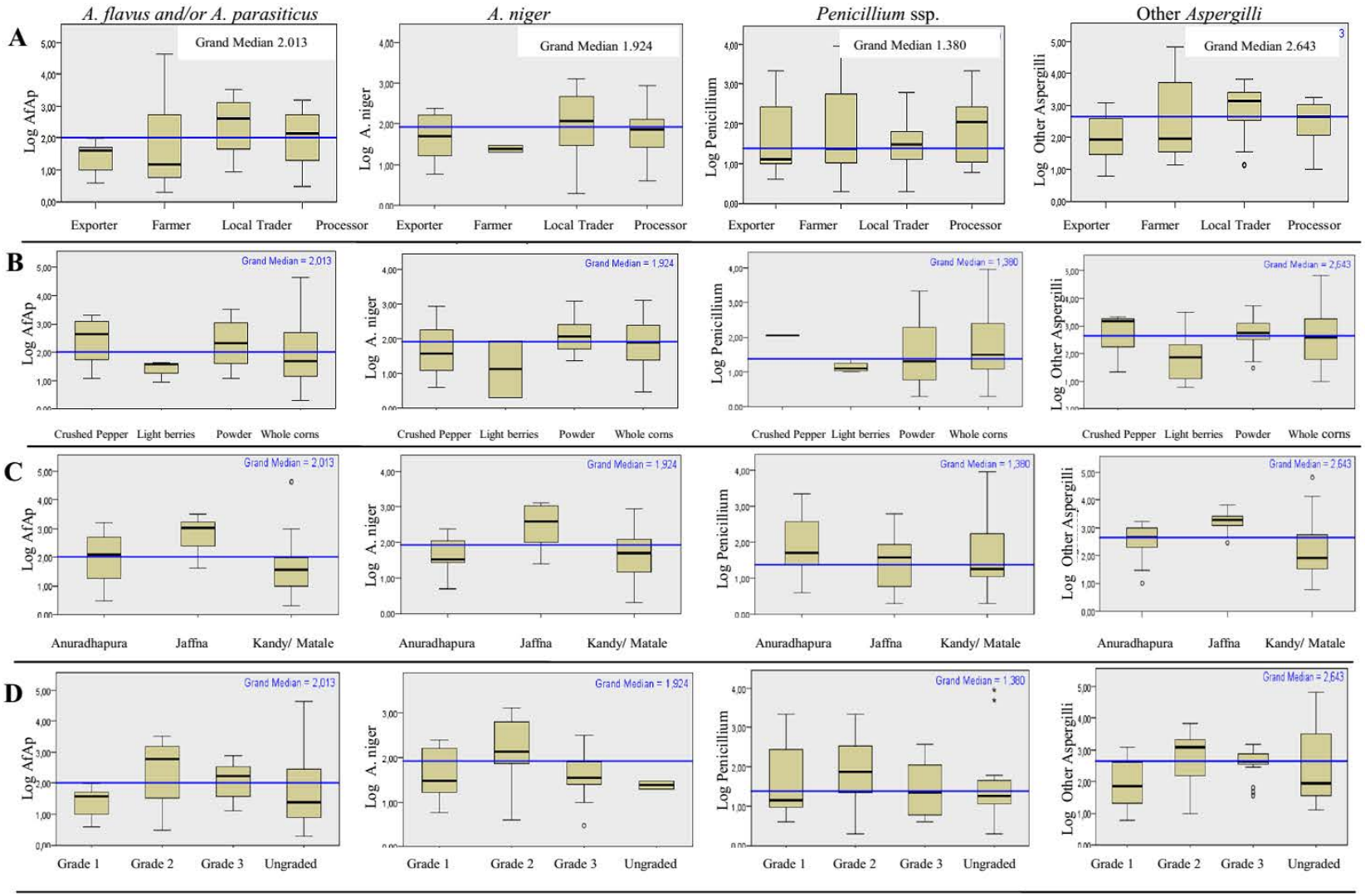

Fig. 3-3. Box and whisker plots showing the comparison of log counts of *Aspergillus flavus* and/or *Aspergillus parasiticus* (AfAp), *Aspergillus niger*, *Penicillium* spp. and other *Aspergillus* among different A) steps of the production chain, B) pepper forms, C) sampling regions and D) pepper grades.

Fig. 3-4. Comparison of the frequency of mould contamination among black pepper samples based on, A) Steps of the production chain, B) Forms of pepper (Crushed pepper: C. pepper; Pepper powder: P. powder; Whole peppercorns: W. corns; Light berries: L. berries), C) Regions and D) Grades of pepper. The frequencies (%) are shown in y-axis.

Similar counts were found as for the samples from the dry region Anuradhapura. However, the mean a_w of the samples from Kandy and Matale were relatively higher (0.70±0.06); this could be due to the higher rainfall and humidity. The higher temperatures and drought conditions also may favour *A. flavus* over other fungi because of its ability to grow on substrates with low a_w (CAST, 2003). When comparing the frequency of AfAp and *A. niger* contamination, they were comparatively higher in the samples from the two dry zones (70-80%) than from the wet zone (50%). A different tendency has been observed with the *Penicillium* occurrence which was higher in the wet zone samples. Probably, rather cooler weather conditions prevailing in these wet regions favour its growth (Peter, 2006).

Comparing the different grades of pepper (Fig. 3-3D) significantly lower counts were observed in grade 1 for AfAp ($p=0.017$) and other *Aspergillus* ($p=0.018$). However, the mean a_w of grade 1 is higher than that of grade 2 and 3 (Table 3-1). These results explain that the mould counts are highly unpredictable and highly variable, regardless of the available water for its growth. It should be stressed that the information on pepper grades was collected verbally from the traders. The widespread prevalence of different moulds in peppers indicate the potential for mycotoxin contamination and could possess a potential health hazard.

3.3.3. Mycotoxin contamination in peppers

The descriptive statistics of each of the mycotoxins in black pepper are shown in Table 3-3 and the frequency of contamination is shown in Fig. 3-5. AFG2, AFG1, AFB2, AFB1, FB1, OTA, STERIG and CIT were among the detected mycotoxins. Considering the AFs contamination, 20% of the black pepper samples were contaminated at least with one of the AFs (<LOQ to 17.3 µg/kg), though 73% of them were contaminated with AfAp (Fig. 3-4 and Fig. 3-5). Contamination of black pepper with AFs and OTA has been previously reported by several researchers at various levels (Saxena and Mehrotra, 1989; Jalili et al., 2010; Zinedine et al., 2006; Bokhari, 2007; Fazekas et al., 2005). In total, 63% of the AFB1 positives exceeded the EU ML of 5 µg/kg. In a study by Romagnoli et al. (2007), none of the black pepper samples were contaminated by AFs. It has been stated that *A. flavus* grows well on the spices but the production of AFs is lower than in cereals (MacDonald and Castle, 1996). The frequency of occurrence of other AFs other than AFB1 was low (<10%) and at low concentrations (<LOQ to 8.4 µg/kg). It could be that not all the *A. flavus* strains are aflatoxigenic on this kind of matrix (Elshafie et al., 2002). According to Banerjee et al. (1993), 100% of the isolates from black pepper were aflatoxigenic. Similarly, Martinez-Magana et al. (1989) identified AFs from all the *A. flavus* strains found in black pepper and white pepper. A high incidence of aflatoxigenic strains (77%) is usually found among *A. flavus* strains (Lisker, 1993). One sample of white pepper was found to be contaminated with either AFB1 (4.9 µg/kg), AFB2 (<LOQ) or AFG2 (5.6 µg/kg).

Table 3-3. Mycotoxin contamination in black pepper, Sri Lanka. The concentrations are given in µg/kg.

Descriptive statistics of positives (n=82)	AFG2	AFG1	AFB2	AFB1	FB1	OTA	STERIG	CIT	Total AFs
Average	7.3	6.6	<LOQ	9.1	122.7	30.9	15.4	89.7	10.3
Median	7.9	NA	<LOQ	5.3	129.9	16.3	12.5	85.8	5.7
Stdev	1.5	NA	NA	5.4	17.7	31.8	9.8	12.9	8.3
Maximum	8.4	NA	<LOQ	17.3	134.5	79.0	49.0	112.0	25.2
Minimum	<LOQ	NA	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
25th percentile	6.9	NA	<LOQ	5.0	119.9	8.3	10.3	85.8	5.0
75th percentile	8.2	NA	<LOQ	13.1	132.6	51.7	14.4	86.3	11.1
90th percentile	8.3	NA	<LOQ	16.0	133.7	71.0	22.5	101.8	23.9
Inter Quartile Range (IQR)	1.4	NA	NA	8.1	12.7	43.4	4.1	0.49	6.1

As shown in Fig. 3-5, STERIG (<LOQ to 49 µg/kg) was very detected frequently in both black pepper (43%) and white pepper (45%) samples. Such higher incidence of STERIG in peppers has been reported for the first time in this study. STERIG, the latest intermediate in the metabolic pathway of AFs biosynthesis, is a possible human carcinogen (IARC, 1993a). Apart from *A. versicolor*, STERIG can also be produced by *A. flavus*, *Aspergillus nidulans*, *Aspergillus rugulosus* and *Aspergillus unguis* (Reijula and Tuomi, 2003). It might be that the pepper components interrupt the biosynthesis of AFs (influence on the production of enzymes) from *Aspergillus* hence, more of the STERIG was frequently found or it was produced by other fungal species like *Penicillium*, *Bipolaris*, *Cheatomium* or *Emiricella* (Samson et al., 2004; Frisvad et al., 2005).

Occurrence of STERIG at high levels in pepper (105 and 125 µg/kg) was previously reported only in an Indian study (Saxena and Mehrotra, 1989). Later studies were focused solely on the two regulated toxins in EU. Notably, EFSA´s panel on contaminants in the food chain (CONTAM) recently assessed the risk for public and animal health related to the presence of STERIG in food and feed (EFSA, 2013) and concluded that more occurrence data on STERIG in food and feed need to be collected to allow dietary exposure assessment.

Next to AFs, OTA has been frequently detected in spices (Jalili et al., 2010). OTA concentration in black pepper was in the range of <LOQ to 79 µg/kg (Table 3-3), but with higher degree of variation, while none of the white pepper samples were contaminated with OTA. In total, 38% of the OTA positives exceeded the EU ML 15 µg/kg. OTA levels of 15 to 69 µg/kg were reported in

black pepper of India (Thirumala-Devi et al., 2001). *P. verrucosum* is an important OTA producing mould in temperate climates like Central and Northern Europe, which grows at 0-31°C and produces OTA at 4-31°C (FAO, 2001). But another OTA producing species *A. ochraceus* was found in few black pepper samples and with very low colony counts. Generally, it is not most frequently found in spices. Hence, the OTA production in Sri Lankan peppers could be due to *Penicillium* or by *A. niger* strain since *Aspergillus carbonarius* was not found in these spices. Abarca et al. (1994) first reported the production of OTA by *A. niger* and isolated the toxin from the culture extract of *A. niger*.

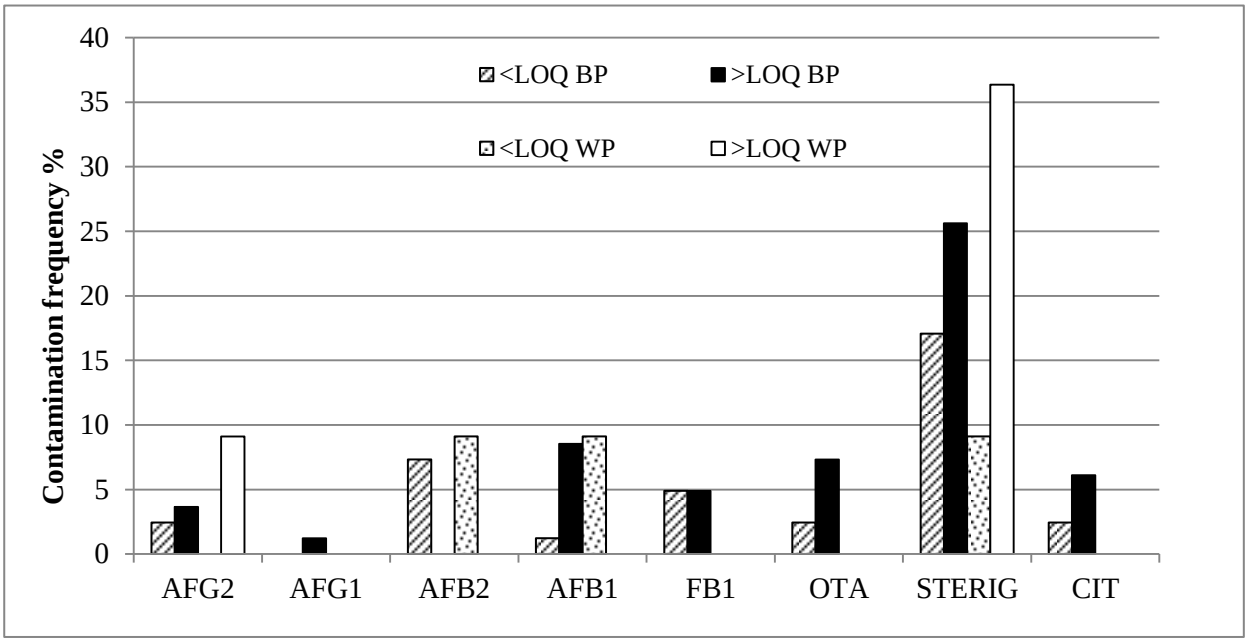

Fig. 3-5. Contamination frequency of different mycotoxins in black (BP) and white pepper (WP).

Furthermore, incidence of FB1 in black pepper samples (10%) is reported for the first time in this study. However, *Fusarium* spp. was not found in pepper samples from Sri Lanka. The a_w of these dry peppers probably were not conducive for *Fusarium* spp. survival. However, they might have been contaminated and produced toxins when the pepper berries were fresh (in field or during initial drying stages) and lately the fungal species probably have died due to inappropriate conditions. Occurrence of the field fungi *Fusarium* spp. is previously reported by Hashem and Alamri (2010) in several spices but not in pepper. *F. moniliforme* and *F. solani* were isolated from all the developmental stages of Indian pepper, however, with low incidence on processed berries (Banerjee et al., 1993). The same species were also isolated by Bokhari (2007). Moreover, some of the isolates of *A. niger* from black pepper were found to be FB2 producers in MEA (Fig. 3-6), however FB2 was not found in the natural samples. May be the FB2 has acted as a biogenic precursor to FB1 (yet, the occurrence of FB1 was very low in the samples) (Desjardins et al., 1996) or there might be some effect of pepper in FB2 production by *A. niger*. This needs to be further studied.

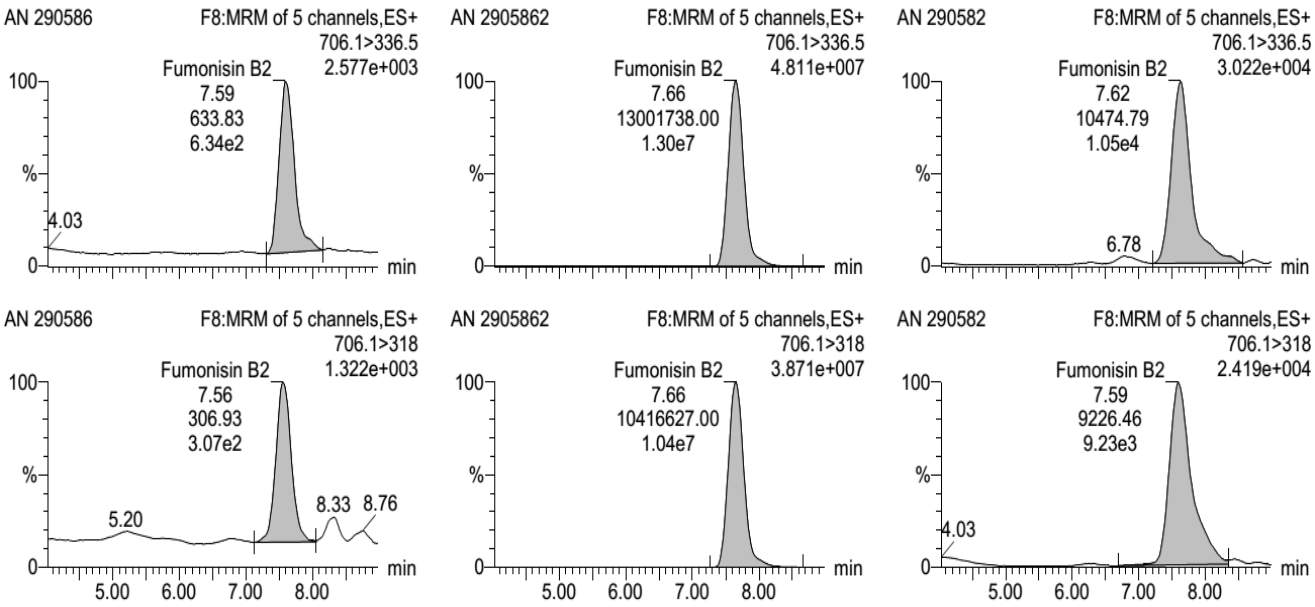

Fig. 3-6. Fumonisin B2 (FB2) production by three *A. niger* isolated from black peppers.

Moreover, CIT was also found in 8.5% of the black pepper samples with a mean concentration 90 µg/kg of positive samples. CIT has also been produced by *Aspergillus* spp., but the major producers are *P. expansum*, *P. verrucosum* and *P. citrinum* (Saxena and Mehrotra, 1989; Frisvad, 2004). Overall, 63% of the black pepper samples were contaminated with at least one mycotoxin (Table 3-4). In 12% of the black pepper samples, more than two toxins were found while six samples were contaminated with more than three toxins. Occurrence of CIT, ZEN and STERIG in black pepper was earlier reported only in an Indian study which used separate methods for each type of toxin (Saxena and Mehrotra, 1989). Moreover, no significant correlations were found between different moulds or between moulds and their mycotoxin production.

Table 3-4. Co-occurrence of mycotoxins in black pepper

Parameter	No of mycotoxins per sample				
	0	1	2	3	4
No of samples	30	35	10	6	1
Frequency (%)	37	43	12	7	1

3.3.3.1. Comparison of Sri Lankan mycotoxins data with the contamination of pepper samples collected in Belgium

Black pepper (n=20) and white pepper (n=7) samples collected from different exotic shops and supermarkets in Belgium were analysed for multiple mycotoxins. Among them, only one black pepper sample was contaminated with AFB1 (<LOQ). Remarkably, 40% of them were contaminated with STERIG in the range of 7.7-77.1 µg/kg, which is similar to the Sri Lankan samples. One white pepper sample imported from Sri Lanka was contaminated with STERIG at

52.8 µg/kg. CIT was found in three white pepper samples with a mean concentration of 199±35 µg/kg. The lesser occurrence on AFs contamination and the absence of OTA in peppers could be due to the stringent regulations practiced in EU.

3.3.4. Exposure assessment to mycotoxins due to pepper consumption

According to Tanaka et al. (2008), black pepper intake in Sri Lanka is 34 g/month/head. However, the sample size was too small (n=15 families) to consider it as a reliable measure of black pepper intake of Sri Lankan population. Therefore, consumption data of other countries on spices were additionally used for exposure assessment since spices from Sri Lanka are also exported to many countries including EU and USA. The deterministic exposure to AFB1, total AFs and OTA due to the intake of Sri Lankan pepper was compared with that of different countries (Table 3-5). The consumption data presented for some countries are of total spices intake since data on peppers alone were not available, therefore the calculations on the exposure should be treated carefully.

Table 3-5. Dietary exposure to AFB1, total aflatoxins and OTA due to the consumption of Sri Lankan black pepper (BP) (ng/kg BW/day); comparison with the intake of different countries (mean and maximum concentrations of each toxin obtained from this study are given within brackets in µg/kg). 60 kg was used as a reference value for head to body weight (BW) conversion.

Country	Dietary intake (Black pepper or spice)	Exposure using mean concentration of positives			Exposure using maximum concentration		
		AFB1 (9.1)	Total AFs (10.3)	OTA (30.9)	AFB1 (17.3)	Total AFs (25.2)	OTA (79)
Sri Lanka	BP: 1.13 g/head/day (Tanaka et al., 2008)	0.17	0.19	0.58	0.46	0.67	2.10
India	BP: 1.53 g/head/day (Tanaka et al., 2008)	0.23	0.26	0.79	0.44	0.64	2.01
New Zealand	BP: 0.21 g/head/day (Fowles et al., 2001)	0.03	0.04	0.11	0.06	0.09	0.27
Thailand	Spice: 14.7 g/head/day (Tantipopipat et al., 2010)	**2.23**[a]	**2.51**	7.58	**4.24**	**6.18**	**19.4**
EU	Spice: 0.5 g/head/day (Fowles et al., 2001)	0.08	0.09	0.26	0.14	0.21	0.66
USA	Spice: 7.7 g/head/day (Fowles et al., 2001)	**1.20**	**1.33**	4.01	**2.24**	**3.26**	10.23
	Spice: 4 g/head/day (ASTA)	0.61	0.68	2.06	**1.15**	**1.68**	5.27

[a] Values exceeding the TDI are shown in bold figures.

A provisional maximum tolerable daily intake (PMTDI) of 1 ng/kg BW/day (Kuiper-Goodman, 1998) and a tolerable daily intake (TDI) of 120 ng/kg BW/week (EFSA, 2006) are fixed for AFB1 and OTA, respectively. According to the existing data on black pepper consumption from Sri Lanka and India, the level of exposure was below the TDI even at higher concentrations of AFs and OTA. The AFB1 exposure in Thailand (2.23 ng/kg BW/day) exceeds by more than two fold the TDI, however the consumption data was for a combination of several spices. Moreover, OTA exposure (19.4 ng/kg BW/day) exceeded the TDI at high concentration. Considering the US consumption of 4 g/person/day (ASTA), exposure was less than the TDI at mean AFB1 concentration. It is very essential to have reliable consumption data of all spices consumed to perform a risk assessment in a systematic way for a particular region. According to the existing data, the spice consumption in EU is very small hence, the mycotoxin exposure can be considered to be well below the proposed TDI.

Sri Lankan cooking quite frequently makes use of black pepper as a main spice in some curry preparations. However, the chilli consumption is far higher than that of pepper in Sri Lanka, and in many other South East Asian countries (Tanaka et al., 2008). Furthermore, the AFs and OTA are often detected at high levels in chilli as well (Santos et al., 2010 and 2011; Saxena and Mehrotra, 1989; Romagnoli, et al., 2007). Hence, the probable exposure to AFs through the intake of chilli could be more significant than pepper. Therefore, in addition to the intake of other food commodities, the consumption of individual spices should be of concern regarding the dietary exposure to aflatoxins and OTA.

3.4. CONCLUSIONS

The study revealed that there is a heavy contamination of mycotoxigenic moulds associated with Sri Lankan peppers regardless of their low water activity and strong anti-microbial property. High overall fungal contamination, high incidence of potential producers of mycotoxins and the presence of different mycotoxins show that peppers can be a means of contamination of food and a risk for consumers health. The multiple-mycotoxins analysis shows that apart from the "classical" mycotoxins (AFs and OTA) of spices, the mycotoxins STERIG, fumonisin B1 and CIT were also found to be present. High frequency of STERIG in peppers and the absence of maximum limits should never be neglected, since its toxicity is similar to the regulated AFB1. Despite the high piperine content in Sri Lankan peppers, the substrate supports the growth of different toxigenic moulds. No correlation could be found between the moisture content or the water activity of the samples and mould or mycotoxin contamination. Controlling the water activity and moisture content at one point of the supply chain cannot be a single solution to ensure the final product safety, but this needs to be followed up in all the steps along the farm-to-fork chain. Complete elimination of mycotoxins or mould growth could be practically impossible even in these dry food

products. Post-harvest practices also need to be supplemented with control strategies like, elimination or detoxification using gamma-irradiation, however this will add cost and there are EU regulations for such applications. Although spices are present in foods in small amounts, they are recognized as important carriers of microbial contamination mainly because of the sub-standard conditions prevailing in the developing countries where they are produced. Peppers are often added to food directly while dining, hence chances of exposure to the contamination are comparatively higher than those spices added during cooking. According to this study, the deterministic exposure to aflatoxins and OTA by consuming pepper remains harmless considering the existing pepper intake data of Sri Lankan population. Individual spices dietary intake of a representative population together with the contamination levels of different mycotoxins is essential to perform a systemic risk assessment due to consumption of different spices.

The mycotoxin contamination data of pepper presented in this chapter was integrated together with the pepper consumption data and a quantitative risk assessment was performed for the Sri Lankan context as described in **Chapter 5**.

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CHAPTER

4

CO-OCCURRENCE OF MYCOTOXINS IN DRY CHILLI (*Capsicum annum* L.) SAMPLES FROM SRI LANKA AND BELGIUM

CHAPTER 4: CO-OCCURRENCE OF MULTIPLE MYCOTOXINS IN DRY CHILLI (*Capsicum annum* L.) SAMPLES FROM THE MARKETS OF SRI LANKA AND BELGIUM

Summary

A multi-mycotoxin method was applied to screen seventeen chemically divergent mycotoxins from dry chilli samples (*Capsicum annum* L.) collected from the markets in Sri Lanka (n=86) and Belgium (n=35) using a high performance liquid chromatography tandem mass spectrometer. In addition to aflatoxins (<LOQ-687 µg/kg) and ochratoxin A (OTA; <LOQ-282 µg/kg), the chilli samples were also found to be contaminated with sterigmatocystin (STERIG; <LOQ-32 µg/kg), fumonisin B2 (FB2; <LOQ-87 µg/kg), citrinin (<LOQ-2.1 mg/kg) and alternariol methyl ether (70 and 222 µg/kg). AFB1 was the predominant mycotoxin contaminating almost 77% of the Sri Lankan samples. While OTA was found in 41% of the samples notably, 38% of them were co-contaminated with STERIG. Overall, 87% and 63% of the Sri Lankan and Belgian samples, respectively were contaminated with at least one mycotoxin. Remarkably, 67% of the Sri Lankan samples exceeded the EU maximum level (ML) of 5 µg/kg for AFB1 and 44% of the samples exceeded the EU ML of 10 µg/kg for total aflatoxins. One third of the Sri Lankan chillies were contaminated with more than three different mycotoxins. Co-occurrence of different mycotoxins, AFB1-OTA (36%), AFB1-STERIG (28%), OTA-AFB1-STERIG (17%) and AFB1-FB2 (14%) was found in different forms of chillies. Higher frequency of mycotoxins co-occurrence found in the processed chillies such as flakes and powder could be due to the fraudulent use of low quality grade chilli pods for processing. Noticeably, 9 of the 11 positive chilli samples from Belgium exceeded the EU ML for AFB1.

Keywords: *Mycotoxins, chilli, Sri Lanka, Belgium, co-occurrence*

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4.1. INTRODUCTION

Chilli (*Capsicum annum* L.) belonging to the night shade family of Solanacea is the second largest consumed spice throughout the world, after the black pepper. The hot sensation of chilli has attracted vast consumers all over the world, especially from South East Asia and Latin America. Chillies are an integral part of the Sri Lankan diet and a major portion is imported from India (CRN India, 2013).

Climatic conditions and lack of good agricultural and manufacturing practices (poor collection conditions, unpretentious production processes, extended drying times in open air and poor storage practices) are of great concern in developing countries where chillies are grown. Moreover, chilli is highly hygroscopic, making it highly susceptible for fungal growth and subsequent mycotoxin production (Banerjee et al., 1993; Banerjee & Sarkar, 2003). Mycotoxin contamination is becoming one of the most insidious challenges to food safety, evidenced by the constant international attention they draw due to their harmful impact on health and economy.

Among various spices, chilli has been extensively reported to be frequently contaminated mainly with aflatoxins (AFB1, AFB2, AFG1 and AFG2) and/or OTA (Table 4-1). The worldwide occurrence of these toxins in different chilli or red hot/chilli pepper forms reported by some countries since the year 2000 is presented in Table 4-1. The level of different mycotoxins in chillies varies in each country as well as in different regions of the country depending mainly on the environmental conditions where it was grown and the storage and/or transportation conditions. Despite the growing economic reputation of chillies as a globally traded food commodity, surprisingly very little information exists on the occurrence of mycotoxins in Asia (Cho et al., 2007; Jalili & Jinap, 2012; Iqbal et al., 2010; Reddy et al., 2001; Tahira et al., 2012).

Consumption of contaminated chillies is detrimental to human health and compromised commercial potential could severely restrict exports. On the basis of these facts, most countries have set regulatory requirements as explained in the introduction part of **Chapter 2**.

Chilli samples were evaluated only for mycotoxins and mould study was not performed in chillies. Mould study and development of mycology models were performed only for peppers considering the fact that pepper is produced and exported from Sri Lanka (chilli is considered as an import issue) and lack of studies in peppers (more details in **Chapter 10**).

Table 4-1. Occurrence of mycotoxins (total aflatoxins, AFB1 and OTA) in different forms of chillies/red chilli peppers (*Capsicum* spp.) in different countries.

Country	Chilli or chilli pepper forms	Total number of samples	Mycotoxin (range or mean±SD in µg/kg)	Positive samples (%)	Clean-up/analytical method	References
Hungary	Ground red pepper	70	AFB1: 6.1-15.7 (0.1[a])	18 (13)	IAC/HPLC-FD	Fazekas et al., 2005
			OTA: 0.4-66.2 (0.2)	32 (22)		
Hungary	Chilli	5	AFB1: 0.75-8.1 (0.1)	2 (40)	IAC/HPLC-FD	Fazekas et al., 2005
			OTA: 2.1 (0.2)	1 (20)		
Turkey	Red pepper powder	100	AFB1: <LOQ-40.9 (0.025*)	68 (68)	ELISA	Aydin et al., 2007
Turkey	Deep-red ground pepper	75	AFB1: 0.11-24.7 (0.025*)	72 (96)	ELISA	Ardic et al., 2008
Turkey	Chilli powder	15	AFB1: 1.6-80.4 (0.4)	15 (100)	IAC/HPLC-FD	Bircan, 2005
Turkey	Red scaled pepper	30	AFB1: 1.9-35.5 (1.0*)	13 (43)	IAC/HPLC-FD	Colak et al., 2006
	Red pepper	30	AFB1: 2.9-11.2 (1.0*)	11 (37)		
Turkey	Red chilli flakes	24	AFB1: 0.13-11.4 (0.16)	19 (79)	IAC/HPLC-FD	Ozbey & Kabak, 2012
			OTA: 0.46-53 (0.19)	18 (75)		
	Red chilli powder	22	AFB1: 0.2-35.7 (0.16)	14 (64)	IAC/HPLC-FD	Ozbey & Kabak, 2012
			OTA: 0.8-98.2 (0.19)	12 (55)		
Spain	Chilli	11	AFB1: 1.4-64.4 (0.375)	NA	IAC/HPLC-FD	Santos et al., 2011
			AFs: 1.9-65.7 (1.5)	11 (100)	IAC/HPLC-FD	
			OTA: 4.3-19.1 (0.3)	11 100	HPLC-FD	
Spain	Chilli	35	AFs: <LOQ-2.49 (0.50)	14 (40)	IAC/HPLC-FD	Santos et al., 2010
			AFB1: <LOQ-0.84 (0.125)	NA	IAC/HPLC-FD	
			OTA: <LOQ-44.6 (0.1)	35 (100)	HPLC-FD	
Ireland	Chilli	30	AFB1: 9.05±8.67 (0.2)	10 (33)	IAC/HPLC-FD	O'Riordan & Wilkinson, 2007
			AFs: 3.23±0.21 (NA)	NA		
Italy	Hot pepper whole/smashed	11	AFB1: 0.57-26.9 (1.5)	5 (45)	IAC/HPLC-FD	Romagnoli et al., 2007
Portugal	Chilli	8	AFB1: 1-5 (1.0*)	3 (38)	IAC/HPLC-FD	Martins et al., 2001
India	Chilli	182	AFB1: 2.02-969 (NA)	102 (59)	HPLC-FD	Reddy et al., 2001
Pakistan	Ground chilli	9	AFB1, AFB2: 6.8-96.2 (NA)	9 (100)	MycoSep/HPLC-FD	Paterson, 2007
	Chilli pods	4	AFB1, AFB2: 0.1-6.6 (NA)	4 (100)	MycoSep/HPLC-FD	
Pakistan	Chilli	40	AFB1: 1.0-30.8 (0.5)	24 (60)	MycoSep-HPLC-FD	Iqbal et al., 2011a
Pakistan	Chilli whole	78	AFB1: 17.4±0.5 (0.5)	26 (33)	HPLC-FD	Iqbal et al., 2011b
	Chilli ground	78	AFB1: 18.5±0.6 (0.5)	31 (41)	HPLC-FD	Iqbal et al., 2011b
Pakistan	Red chilli	15	OTA: 23-94 (1.0)	13 (80)	IAC/HPLC-FD	Tahira et al., 2012
Korea	Red pepper flour	41	AFB1: 0.08-4.45 (0.03)	7 (17)	IAC/HPLC-FD	Cho et al., 2007
Malaysia	Red chilli	80	AFB1: 0.2-56.6 (0.06)	52 (65)	IAC/HPLC-FD	Jalili and Jinap, 2012
			OTA: 0.2-101 (0.06)	65 (80)	IAC/HPLC-FD	

NA-Not available; LOQ-Limit of quantification; IAC-Immuno affinity column; FD-Fluorescence Detector; ELISA-Enzyme Linked Immuno-Sorbent Assay; AFs-Sum of all aflatoxins (AFB1+AFB2+AFG1+AFG2), [a]LOQs are given within brackets, * LOD.

Except aflatoxins and OTA, determination of other mycotoxins in chillies has not been carried out due to lack of multi-analyte methods. So far, there were no studies on the co-occurrence of several mycotoxins in chillies from Sri Lanka or from Belgium, despite its high consumption in Sri Lanka and its heterogenic use in Belgium. Hence, the present investigation aims in determining different mycotoxins in chillies from the markets in Sri Lanka and Belgium. This is the first ever study to apply a multi-analyte method to simultaneously quantify different co-occurring mycotoxins in chillies.

4.2. MATERIALS AND METHODOLOGY

4.2.1. Chemicals and reagents

Magnesium sulphate anhydrous was purchased from Sigma-Aldrich, Steinheim, Germany. Formic acid ULC-MS grade (99%) was supplied by Bio Solve BV (Dieuze, France). All other chemicals and reagents used for LC-MS and for sample preparation were of analytical grade; same as described previously in **Chapter 2 (section 2.2.1).**

4.2.2. Mycotoxin standards

Mycotoxin reference standards used were the same and the working standard solutions were prepared similarly as described in **Chapter 2 (section 2.2.2).**

4.2.3. Sample collection

In total 121 chilli samples were collected from various markets in Sri Lanka (n=86) and Belgium (n=35) during the period 2012-2013. Different forms of chilli samples collected from Sri Lanka included, whole chilli pods (n=18), chilli powders (n=42) and chilli flakes or crushed chillies (n=26). Among the 18 whole chilli pods, 16 were imported from India and two were from China. Samples from Sri Lanka were collected from small groceries and supermarkets in Jaffna (n=42), Anuradhapura (n=14) and Colombo (n=30). Samples collected from open markets in Sri Lanka were packed air tight in low density polyethylene and transported to the Laboratory of Food Analysis in Belgium. Samples from Belgium (whole chilli pods (n=3), chilli powders (n=27) and chilli flakes (n=5)) were collected from exotic shops and supermarkets in the region of Ghent (n=32) and Antwerp (n=3). The samples collected from the exotic shops (n=33) were imported from Lebanon (n=10), Thailand (n=8), China (n=4), India (n=2) and one each from Turkey, Uganda and Vietnam. Eight samples collected from these shops were without the information on the origin of the produce. Two samples were collected from Belgian supermarkets. Whole chilli pods and chilli flakes were finely ground using a universal grinding mill (M20 IKA®-WERKE; Staufen, Germany) prior to the analysis. The samples were stored at 4°C until analysis.

4.2.4. Extraction of samples for mycotoxins analysis

Samples were extracted using the QuEChERS based approach as described in **Chapter 2 (section 2.2.4).** The instrumentation and the conditions were the same as described in **Chapter 2 (section 2.2.5).**

4.2.5. Statistical analysis

The Kolmogorov-Smirnov (K-S) and/or Shapiro Wilk test together with the corresponding Q-Q plots were used to determine the normality of the contamination data distribution. Due to the non-normal distribution of the data, the non-parametric Kruskal-Wallis one way ANOVA was used to assess the significance and post-hoc pairwise multiple comparisons were made to identify the differences among the variables upon significant results. The statistical analyses were carried out using the SPSS statistical package (IBM®, Version 21). Level of confidence was 0.05 unless otherwise specified.

4.3. RESULTS AND DISCUSSION

4.3.1. Mycotoxin contamination in chillies from Sri Lanka

In addition to the frequently found aflatoxins and OTA, occurrence and co-occurrence of several other mycotoxins STERIG, FB2, CIT and AME in chillies are reported for the first time in this study.

4.3.1.1. Aflatoxins

An overview of the mycotoxins detected in Sri Lankan chillies is shown in Table 4-2. Remarkably, 87% of the samples were contaminated at least with one mycotoxin. Overall, 77% of the total samples (Fig. 4-1) were contaminated with AFB1 with mean (±SD) concentration of positives 15.6±11.6 µg/kg. Notably, 67% of them exceeded the EU ML of 5 µg/kg. Among AFs, AFB1 was very frequently found in the chilli samples analysed and the second most frequent aflatoxin was AFB2. Interestingly, the highest contamination of AFB2 was found in a whole chilli and a chilli flake sample (31.5 and 6.1 µg/kg, respectively) that were also contaminated with the highest levels for AFB1 (687 and 91.3 µg/kg, respectively). The whole chilli sample which had the highest contamination (687 µg/kg) was not included in the mean calculation. Our data shows that, 39, 92 and 83% of the whole chilli, chilli flakes and chilli powder samples, respectively, were contaminated with AFB1 (Table 4-2). This indicates that there could be a potential risk in developing aflatoxin induced liver cancer due to the highly contaminated chilli consumption in Sri Lanka.

Frequency of contamination with AFB2 was 10%, while only one sample was positive for AFG1 or AFG2 (Table 4-2). Therefore, the contribution of AFB2, AFG1 and AFG2 to the total AFs was very small compared to AFB1. According to Paterson (2007), AFG1 and/or AFG2 were not detected in any of the chilli samples (n=13) from Pakistan. Very low levels of AFG1 and AFB2 were reported in chilli samples from Spain (Santos et al., 2010). Moreover, 44% of the positive samples exceeded the EU ML of 10 µg/kg for total AFs in our study. Also, 12% of positive samples exceeded the Sri Lankan standard of 30 µg/kg for total AFs. For comparison reasons, aflatoxin contaminations reported in various countries are summarized in Table 4-1.

Comparing different forms of chillies, higher frequency of AFB1 or total AFs was found in processed chilli samples (chilli flakes (92%) and chilli powder (83%)) compared to the whole chilli pods (39%). AFB1 contamination was found to be higher than the EU ML in 33, 69 and 48% of the whole chilli, chilli flakes and chilli powder, respectively. Moreover, total AFs level exceeded the EU ML in 28% of the whole chillies, 54% of the chilli flakes and 21% of the chilli powder (Table 4-2). Frequency of total AFs contamination in chilli flakes was more than twice compared to that of powder and whole chilli pods. Also in Pakistan, highest contamination of aflatoxins were found in ground chilli samples compared to whole pods (Paterson, 2007; Iqbal et al., 2010). The concentration of AFB1 ($p=0.012$) and total AFs ($p=0.008$) were found to be significantly different among different forms of chilli ($\alpha=0.05$). Among the different forms, whole chilli pods were the least contaminated with these mycotoxins. A high contamination in the two processed forms, chilli powders and flakes could be due to the fraudulent usage of low quality chilli pods for processing or poor packaging and storage conditions of these products later on. Hence, damaged pods with visible fungal contamination should be discarded. It is also possible that, upon inappropriate packaging the chilli flakes and chilli powder could adsorb more water from the environment compared to the whole chilli pods due to their high surface area.

Comparing the mycotoxin contamination among different regional markets in Sri Lanka, no significant differences were observed in AFB1 or total AFs contamination ($p>0.05$). It should be mentioned that the whole chilli pods in Sri Lanka are generally stored in jute bags which could allow the pods to adsorb humidity. They are generally sold in small groceries exposed to air and dust. Hence, many kinds of fungi could contaminate chillies, including the most potent *Aspergillus* spp. which has the optimum temperatures of 27-38°C and relative humidity of 85% for growth (Williams et al., 2004).

Table 4-2. Occurrence and contamination levels (µg/kg) of multiple mycotoxins in different forms of chilli samples from Sri Lanka (n=86).

Form of chilli	Descriptive statistics	Mycotoxin contamination								
		AFG2	**AFG1**	**AFB2**	**AFB1**	**AFs[b]**	**OTA**	**FB2**	**STERIG**	**CIT**
Whole chilli pods (n=18)[a]	Number of positives[c] (%)	0	0	1 (5)	7 (39)	7 (39)	2 (11)	1 (6)	5 (28)	4 (22)
	Range	NA[d]	NA	31.5	<LOQ[e]-687	<LOQ-718	<LOQ-5.3	NA	<LOQ-31.8	<LOQ-2107
	Mean of positives	NA	NA	31.5	133.1	138.4	NA	87.1	34.3	1363
	Mean of total	NA	NA	1.75	44.5	46.2	NA	4.84	4.57	170.3
	Median	NA	NA	0	22.9	22.9	0	0	34.3	1362
	No. of samples >EU ML	NA	NA	NA	6	5	0	NA	NA	NA
Chilli flakes (n=26)[a]	Number of positives (%)	0	1	6 (23)	24 (92)	24 (92)	13 (50)	9 (35)	10 (39)	1 (4)
	Range	NA	<LOQ	<LOQ-6.1	<LOQ-91	<LOQ-97.4	<LOQ-15	<LOQ	<LOQ	<LOQ
	Mean of positives	NA	<LOQ	6.1	20.5	20.8	12.6	<LOQ	<LOQ	NA
	Mean of total	NA	NA	0.29	16.6	16.3	4.9	NA	NA	NA
	Median	NA	0	6.1	16.9	16.9	11.9	0	0	NA
	No. of samples >EU ML	NA	NA	NA	18	14	0	NA	NA	NA
Chilli powder (n=42)[a]	Number of positives (%)	1 (3)	0	2 (5)	35 (83)	35 (83)	20 (48)	3 (7)	18 (43)	2 (5)
	Range	<LOQ	NA	<LOQ	<LOQ-31.2	<LOQ-31.2	<LOQ-282	<LOQ	<LOQ-13.6	<LOQ
	Mean of positives	NA	NA	<LOQ	10.4	10.4	37.8	<LOQ	13.0	<LOQ
	Mean of total	NA	NA	NA	6.6	6.6	16	NA	1.0	NA
	Median	NA	NA	NA	7.4	7.4	13.5	NA	13.4	NA
	No. of samples >EU ML	NA	NA	NA	20	9	2	NA	NA	NA

[a] Number of samples; [b] AFs: Sum of all aflatoxins (AFB1+AFB2+AFG1+AFG2); [c] Number of samples that were quantified (>LOQ) and in between LOD and LOQ (<LOQ), percentage refers to the number of positives divided by the total number of chilli samples of particular form; [d] Not Applicable; [e] LOQ: Limit of quantification.

4.3.1.2. Ochratoxin A

Following AFB1, OTA was the most prominent mycotoxin found in all the chilli samples as it has been previously reported in some other countries (Table 4-1). The results on the OTA occurrence in 86 chilli samples from Sri Lanka are shown in Table 4-2. These results indicate that 41% of the samples were contaminated with OTA in the range of <LOQ to 282 µg/kg. Concentration of OTA was significantly different (p=0.01) among different forms of chilli. OTA concentration in chilli powders was significantly higher than in chilli pods (p=0.009) while a marginal significance in concentration was found with chilli flakes (p=0.046). Moreover, frequency of OTA occurrence in chilli powder (23%) was considerably higher than flakes (15%) or whole chillies (2%). Only, two chilli powders exceeded the EU ML of 30 µg/kg, while no regulation exists for OTA in Sri Lanka. It is important to notify that from 2015 the revised legislation for OTA in chillies will be 15 µg/kg (EC, 2012). Moreover, no significant differences in OTA contamination (p=0.628) were found among the samples collected from the different markets in Sri Lanka.

4.3.1.3. Sterigmatocystin, Fumonisin and Citrinin

Occurrence of STERIG, FB2 and CIT in chillies was reported for the first time in this study (Table 4-2). Quite high frequency of occurrence of STERIG (38%) was found (Fig. 4-1); this is attributable to the high contamination of AFB1 found, since STERIG is the very last intermediate in the aflatoxin biosynthetic pathway (Sweeney and Dobson, 1999).

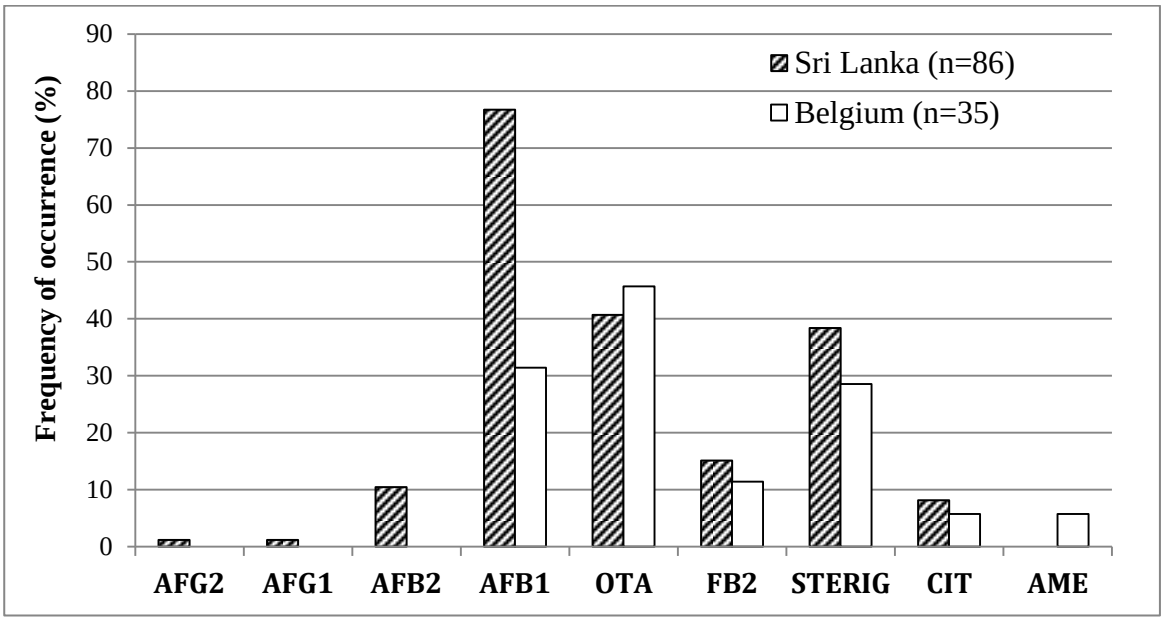

Fig. 4-1. Frequency of mycotoxins occurrence in chilli samples from the markets of Sri Lanka and Belgium.

The concentration of STERIG ranged from <LOQ (11 µg/kg) to 36.8 µg/kg. However, only 15% of the positive samples were quantifiable (mean±SD 21.4±13.6 µg/kg). Previously, STERIG was detected in 25% of the grains (0.7-83 µg/kg) from Latvia (Veršilovskis and De Saeger, 2010).

STERIG is generally recognized as a potential carcinogen, mutagen and teratogen and it is acutely toxic to the liver of most animals tested. It has been classified as a group 2B possible human carcinogen (IARC, 1993a). However, its role in human toxicity is unknown highlighting the importance of more occurrence data to enable risk analysis (Veršilovskis and De Saeger, 2010).

Furthermore, FB2 was found in 15% of the chilli samples. One whole chilli sample had the highest FB2 concentration of 87 µg/kg of all the samples analysed. The FB2 amounts found in chilli flakes and chilli powders were <LOQ (64.2 µg/kg). All fumonisins are carcinogenic; compared to FB1, FB2 has been shown to be more cytotoxic (Gutleb et al., 2002). Furthermore, the nephrotoxic mycotoxin, CIT was found in 8% of the chillies. Remarkably, a very high concentration of CIT was found in a whole chilli pod (2.1 mg/kg). Out of the 18 whole chilli pods, 4 samples were contaminated with CIT, while CIT levels were <LOQ in one chilli flake and two chilli powder samples (Table 4-2). Very small concentrations of DON (<LOQ-0.3 µg/kg) and T-2 (<LOQ-0.2 µg/kg) in 18% of the chilli (n=11) samples were previously reported (Santos et al., 2011). According to Patel et al. (1996), one out of the four *Capsicum* powder samples contained DON (8 µg/kg) and HT-2 (24 µg/kg) while T-2 was not detected. In addition to T-2 and HT-2 toxins, other toxins like, NEO, DON derivatives, FB1 and FB3 were not detected as well in any of the chilli samples in our study. Moreover, there are no legislations exist for any of these toxins in spices in the EU or anywhere in the world.

4.3.1.4. Pre- and post-harvest practices conducive for mould growth and mycotoxin production in chillies in Sri Lanka

Growth of toxigenic fungi and mycotoxin production in chillies possibly can occur in the field during crop production, picking, drying, handling, packaging, storage and transportation or due to physical damage of chilli pods and insect infestation. Chilli produced in Sri Lanka is generally consumed as green. Dry chillies produced by very few small scale farmers are used for their own consumption. Therefore, most of the whole dry chillies are imported from India and China. They are further processed in Sri Lanka at small to large scale processing industries to produce chilli powder and chilli flakes. The very limited number of Sri Lankan farmers who produce dry chillies, usually dry them in the open air under the sun by spreading on bare ground next to the street or on a layer of empty polythene sacks or tarpaulines. Sun drying is also the most common and cheapest method of drying practiced in other South East Asian countries like India and Pakistan, where chilli is produced on a large scale (Paterson, 2007; Reddy, 2001; Iqbal et al., 2010). High contamination of mycotoxins in the present finding especially AFs and OTA in chillies could be mainly due to the warmer conditions prevailing in Sri Lanka which are highly favourable for fungal proliferation and mycotoxin production.

In Sri Lanka, the traders used to sprinkle water to chillies to increase the weight of the pods as a marketing technique, which could create ideal conditions for the growth of many moulds. Moreover, chillies are hygroscopic goods, which can interact with the moisture in air as well. The risk of mould growth is naturally at its greatest in warm and damp air. Therefore, proper packaging is essential to prevent whole pods as well as the processed chilli products from adsorbing water from the air. If chillies have not been properly dried when packaged, mould growth and blackening can occur (lump formation). Self-heating of the product may occur as a result of an excessive intrinsic moisture content or excessive tight packaging. Red chilli pods immediately after harvesting (65-80% moisture content) need to be dried below 10% moisture content to avoid fungal growth. Also storage temperatures below 13°C can reduce the aflatoxin accumulation (Iqbal et al., 2010). However, chillies in Sri Lanka are never cold stored. Hence, mycotoxins contamination in developing country like Sri Lanka could also be due to the lack of infrastructure facilities and inadequate quality control measures along the food chain. Thus, controlling the conditions that favour mycotoxin production is important to guarantee contamination-free products and to reduce the health risks for the consumers in Sri Lanka as well in other countries.

4.3.2. Mycotoxin contamination in chillies from Belgium

Similar to the mycotoxin occurrence in Sri Lankan chilli samples, AFB1 (8.1±1.9 µg/kg) and OTA (9.9±4.3 µg/kg) was the most prominent contaminant in samples from the markets in Belgium, especially exotic shops (Table 4-3). Overall 63% of the samples were contaminated at least with one mycotoxin and 34% had more than two mycotoxins (Table 4-4). Moreover, 74% of the chilli powders (n=27) were contaminated at least with one mycotoxin.

Table 4-3. Occurrence and contamination levels (µg/kg) of multiple mycotoxins in chillies from Belgium (n=35).

Description	AFB1	OTA	FB2	STERIG	CIT	AME
Mean concentration of positives (µg/kg)	8.07	9.93	NA[a]	NA	NA	146
Number of positives	11	16	4	10	2	2
Number of samples >EU ML	9	0	NA	NA	NA	NA
Minimum (µg/kg)	<LOQ[b]	<LOQ	<LOQ	<LOQ	<LOQ	69.7
Median(µg/kg)	7.64	9.98	<LOQ	<LOQ	<LOQ	146
Maximum (µg/kg)	11.9	17.9	53.8	<LOQ	<LOQ	222
No of samples <LOQ	2	6	3	10	1	0
Frequency of samples <LOQ (%)	5.7	17.1	8.6	28.6	2.9	0

[a] Not applicable; [b] Limit of quantification

Table 4-4. Frequency on the number of co-occurring mycotoxins in chilli samples from Sri Lanka and Belgium.

No of toxins per sample	Sri Lanka		Belgium	
	No of samples	Percentage	No of samples	Percentage
0	11	13	13	37
1	22	26	10	29
2	27	31	6	17
3	16	19	3	9
4	9	11	3	9
5	1	1	Not found	-

Notably, 9 out of the 11 positive samples exceeded the EU ML of 5 µg/kg for AFB1; of which 7 samples were products from Lebanese origin and two were from Thailand. Two Lebanese chillies had AFB1contamination higher than 10 µg/kg, the EU ML for total AFs. It is important to state that a significant amount of samples exceeded the ML for AFB1, in an EU country where strict legislations for mycotoxins are enforced. It could be that these samples escaped the regulations or they have been contaminated later due to poor packaging and/or inappropriate storage. Chilli powder imported from India was also contaminated with AFB1 and OTA but below their LOQs, while a crushed chilli sample was positive for CIT. A chilli powder from Turkey was positive for OTA while the whole chilli pods from Uganda were contamination free. None of the samples were contaminated with any other aflatoxins (AFB2, AFG1 and AFG2) (Fig. 4-1). OTA was found in 46% of the samples, while none of the OTA contaminated samples were above the EU ML. Both the samples collected from the Belgian supermarkets were positive for OTA but AFB1 free. One of them was contaminated also with STERIG. STERIG contamination remained also the third similar to Sri Lanka in terms of frequencies. In total, 29% of the samples were positive for STERIG. However, all of them were below LOQ. Furthermore, FB2 (<LOQ-53.8 µg/kg) was found in 11% of the samples while CIT was found in only two samples (<LOQ). AME was detected for the first time in two red chilli samples (Table 4-3). However, they were not detected in any of the Sri Lankan samples because high temperature in Sri Lanka may not favour the growth of *Alternaria* species. Detection of several mycotoxins in chillies from the markets in Belgium indicates that a strict monitoring of the enforced regulations over the imported chilli products is very essential to ensure that the contaminant free food products reach the consumers. Only limited products have official control for mycotoxins during importation according to EC 669/2009 (EC, 2009). Very recently, the rapid alert system for food and feed (RASFF 11.11.2014) has notified (by UK) a very high concentration (20.3 mg/kg) of AFB1 in pepper imported from India.

4.3.3. Co-occurrence of mycotoxins in chillies

Regardless of different forms of chilli, one third of the Sri Lankan samples were contaminated with minimum two mycotoxins. Remarkably, another 30% of the samples were contaminated with more than three mycotoxins. Frequency of different co-occurring mycotoxins in chilli samples collected from Sri Lanka and Belgium are shown in Tables 4-4 and 4-5.

Table 4-5. Frequency of different types of co-occurring mycotoxins in chilli samples from Sri Lanka and Belgium.

Country	Form of chilli (number of samples)	Description	Co-occurrence of mycotoxins			
			AFB1 and OTA	AFB1 and STERIG	OTA, AFB1 and STERIG	AFB1 and FB2
Sri Lanka	Chilli powder (n=42)	Number	20	14	10	3
		Percentage[a]	48	33	24	7
	Whole chilli pods (n=18)	Number	2	3	0	1
		Percentage	11	17	0	6
	Chilli flakes (n=26)	Number	13	11	4	9
		Percentage	50	42	15	35
Belgium	All chilli samples (n=35)	Number	8	6	6	4
		Percentage	23	17	17	11

[a] Percentage refers to the number of samples with certain co-occurrence divided by the total number of chilli samples of the particular form.

Co-occurrence of OTA and AFB1 was found in 41% (35/86) of the Sri Lankan chilli samples. Concentrations of co-occurring AFB2, AFB1, OTA, FB2, STERIG and CIT in different forms of chilli samples from Sri Lankan markets are shown in Table 4-6. There are only very few studies reporting the co-occurrence of AFB1-OTA or AFs-OTA in chillies. A study in Turkey reports the co-occurrence of OTA-AFs in 62.5% of the red chilli flakes and 41% of the red chilli powder samples (Ozbey and Kabak, 2012). In a recent study, 75% of the paprika and 65% of the chilli samples had more than one toxin; further it has been reported that OTA incidence was correlated with the presence of AFB1 and total AFs (Santos et al., 2010). A whole chilli sample contaminated with three mycotoxins (AFB1, STERIG and FB2) and a chilli powder contaminated with five different mycotoxins (AFB2, AFB1, OTA, FB2 and STERIG) in our study are shown in Fig. 4-2.

In total, 33% of the samples were found to be contaminated with both AFB1 and STERIG. Remarkably, 85% of the samples positive for STERIG were also contaminated with AFB1. In total, 16% and 15% of the Sri Lankan samples had a co-occurrence of AFB1-OTA-STERIG and AFB1-

FB2, respectively. It is interesting to note that compared to the whole chilli pods, processed chilli samples had the highest frequency of co-occurrence in any combination of mycotoxins described (Table 4-5). Considering the samples from Belgium, similar co-occurring mycotoxins were found alike the samples from Sri Lanka. In total, 34% of the samples had more than two toxins (Table 4-4). Sixty four percentage of the AFB1 positive samples were also contaminated with STERIG. Moreover, 23% of the total chillies were contaminated with both AFB1 and OTA; while 17% had a co-contamination of the three mycotoxins AFB1-OTA-STERIG. AFB1 and FB2 were found to co-occur in four samples. A chilli powder contaminated with four mycotoxins (AFB1, STERIG, OTA and FB2) and a sample positive for AME are shown in Fig. 4-3.

Table 4-6. Co-occurrence of mycotoxins AFB2, AFB1, OTA, FB2, STERIG and CIT in different forms of chilli samples from Sri Lankan markets (concentrations are given in µg/kg).

Form of red chilli	AFB2	AFB1	OTA	FB2	STERIG	CIT	No of toxins/sample	Origin of market
Chilli powder	ND[a]	18.9	26.5	<LOQ[b]	<LOQ	ND	4	Colombo
Chilli powder	<LOQ	29.7	6.98	ND	<LOQ	ND	4	Colombo
Chilli powder	ND	16.4	7.70	<LOQ	<LOQ	<LOQ	4	Colombo
Chilli powder	ND	31.2	282	ND	<LOQ	ND	3	Anuradhapura
Chilli powder	ND	6.45	15.8	ND	<LOQ	ND	3	Jaffna
Chilli powder	ND	16.9	12.5	ND	<LOQ	ND	3	Jaffna
Chilli powder	ND	7.44	13.5	ND	<LOQ	ND	3	Jaffna
Whole chilli pods	ND	14.9	ND	ND	<LOQ	ND	2	Colombo
Whole chilli pods	ND	45.4	ND	87.1	14.0	<LOQ	4	Jaffna
Whole chilli pods	31.5	687	ND	ND	<LOQ	ND	3	Jaffna
Chilli flakes	<LOQ	23.0	11.9	<LOQ	ND	ND	4	Colombo
Chilli flakes	ND	19.7	ND	<LOQ	<LOQ	ND	3	Colombo
Chilli flakes	ND	43.7	11.6	ND	<LOQ	<LOQ	3	Colombo
Chilli flakes	ND	30.4	4.96	<LOQ	<LOQ	ND	4	Anuradhapura
Chilli flakes	<LOQ	14.1	<LOQ	<LOQ	<LOQ	ND	5	Jaffna
Chilli flakes	ND	22.1	15.0	ND	<LOQ	ND	3	Anuradhapura

[a] Not detected; [b] Limit of quantification

Currently, there are no legislations on combined contamination of mycotoxins in spices except for the control of the four aflatoxins (AFB1, AFB2, AFG2 and AFG1). This study confirms a high frequency of mycotoxin co-occurrences in chillies from Sri Lanka and Belgium. As emphasized by Speijers and Speijers in 2004, a combined intake of different mycotoxins at different concentration levels may lead to a higher risk than their single intakes. Furthermore, it has been shown that OTA

could increase the mutagenicity of AFB1 (Sedmikova et al., 2001) and a synergistic effect was found by Huff and Doerr (1981) due to these combined mycotoxins.

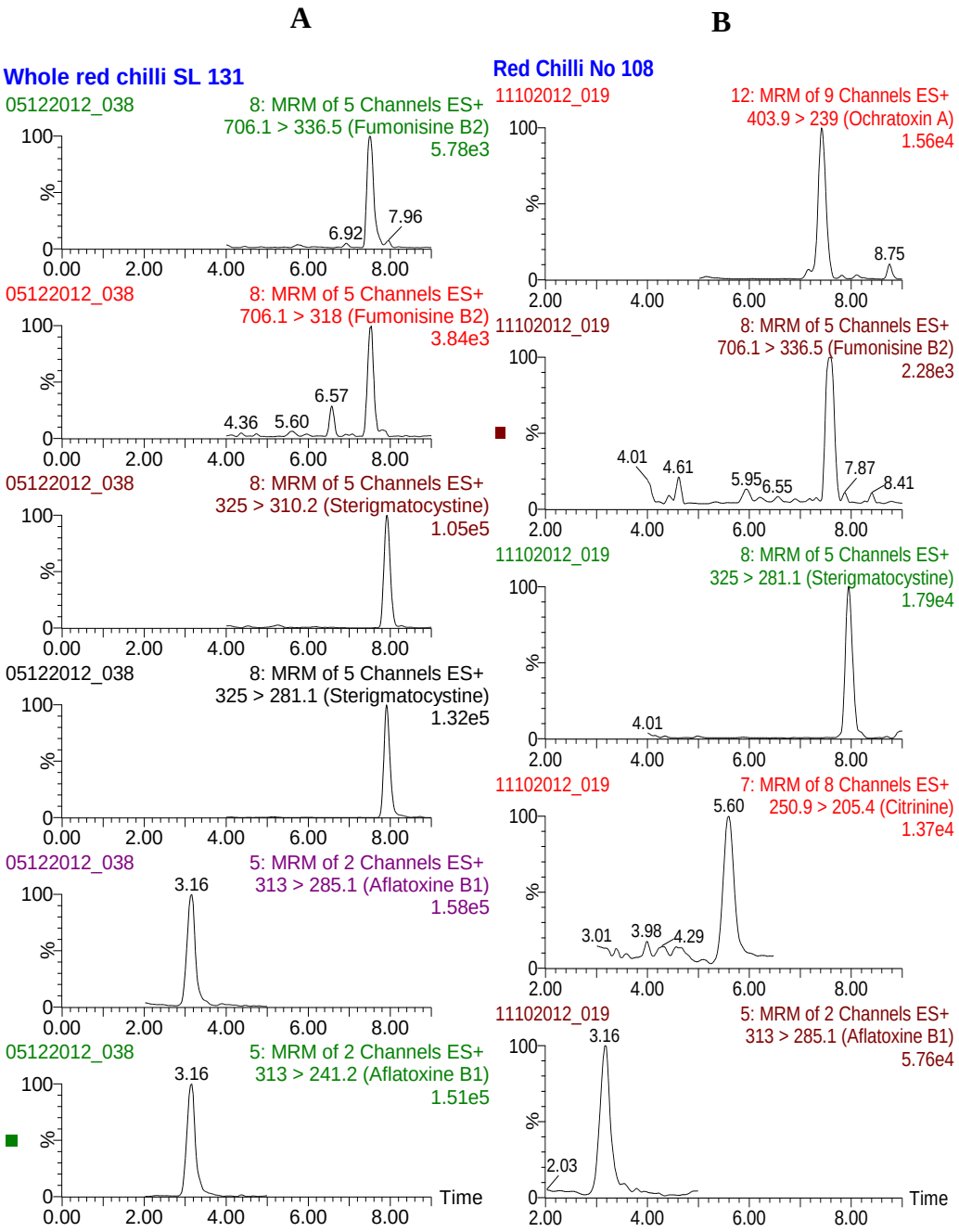

Fig. 4-2. MRM transitions of chilli samples from Sri Lanka showing the natural co-occurrence of different mycotoxins, A) whole chilli pod from Jaffna with FB2 (87.1 µg/kg), STERIG (36.8 µg/kg) and AFB1 (45.4 µg/kg) (Quantification and confirmation transitions for each toxin are shown), B) chilli powder from Colombo with OTA (7.7 µg/kg), FB2 (<LOQ), STERIG (<LOQ), CIT (<LOQ) and AFB1 (16.4 µg/kg) (only one fragment ion per toxin is shown).

In addition to the co-occurrence of AFB1-OTA, this study is the first to report the co-occurrence of several other mycotoxins in chilli. Similar to the synergistic effect of OTA on AFB1 mutagenicity, eventually other co-occurring mycotoxins depending on the type and amount may also execute different levels of toxicity in humans as well as in animals. AFB1 co-occurred with FB1 in food

samples from high-incidence area of human primary liver cancer in China; the resultant co-exposure has been suggested to be a risk factor for the development of the disease (Ueno et al., 1997). According to Gelderblom et al. (2002), cancer initiating potency increased when rats were treated with AFB1 and FB1 in a sequential manner. Several other studies have shown that the combined AFB1-FB1 acted synergistically in cancer initiation and promotion (McKean et al., 2006). However, no toxicological studies were performed with a binary mixture of AFB1 and FB2; in our study a 14% co-occurrence of these two toxins was found in total. Synergistic or at least additive effects of co-occurring OTA and FB1 and the role of OTA and CIT in nephrotoxicity have been studied as well (Tammer et al., 2007). However, OTA co-occurred with CIT only in two Sri Lankan chilli samples. Most of the available studies on mycotoxin mixtures exerted synergistic or additive effects, calling our attention to the significant threat to human health due to the consumption of foods contaminated with several mycotoxins.

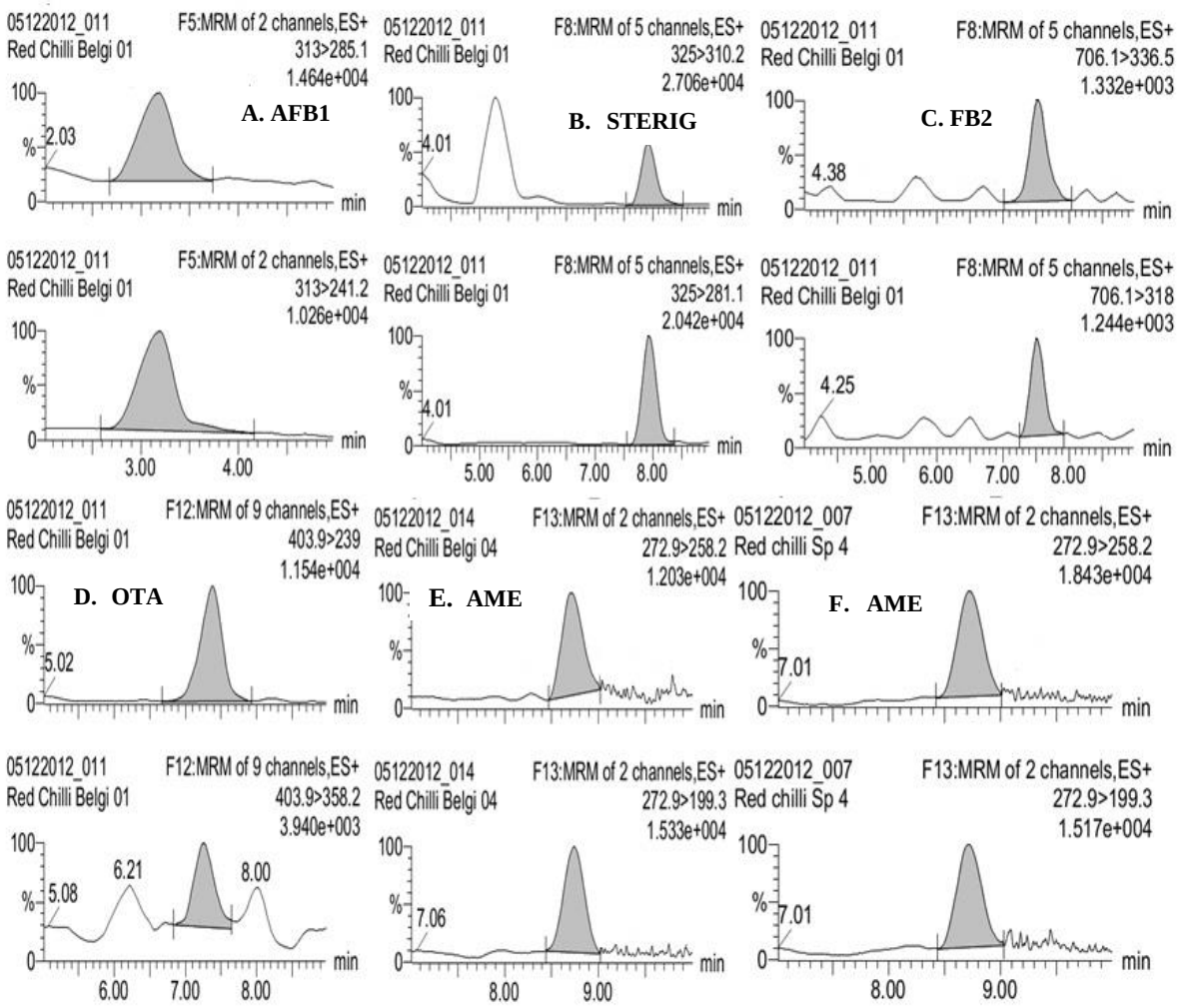

Fig. 4-3. MRM chromatograms showing the co-occurrence of mycotoxins in a chilli powder collected in Belgium (origin Lebanon) contaminated with A) 11.1 µg/kg of AFB1, B) 11.6 µg/kg of STERIG, C) 53.7 µg/kg of FB2 and D) 15.6 µg/kg of OTA, E) a chilli powder contaminated with 222 µg/kg of AME and F) spiked chilli powder with AME at similar concentration of E, shown for comparison. Quantification and confirmation transitions are shown for all the toxins.

In contrast to the laboratory animals, in reality people are frequently exposed to diverse toxic substances (including mycotoxins) present in food. With regard to these chemical hazards, there is an increasing concern over the possible "cocktail effects" of combined exposure due to multiple food related toxic substances over the years. Therefore, in addition to the existing MLs for single toxins, MLs for co-occurring mycotoxins for different food matrices need to be proposed as the overall toxicity could be different (Carpenter et al., 1998). The co-occurrence of two or more mycotoxins in 54% of the total chilli samples in our study highlights the importance of multi-mycotoxin analysis and to assess their overall exposure and combined toxicological effects. Despite the low consumption of spices compared to the staples, the co-occurrence of different mycotoxins should never be underestimated considering their collective toxicological effects.

4.4. CONCLUSIONS

This study shows that chillies are frequently contaminated with several toxicologically significant mycotoxins. Mycotoxin contamination in 87% and 63% of the samples in markets of Sri Lanka and Belgium, respectively emphasizes the importance of good agricultural and manufacturing practices in chilli production and warrants the need for routine analysis to verify these practices. The study reveals cases of samples exceeding the EU maximum limits from shops in Belgium. Developing countries like Sri Lanka and other South East Asian countries where chilli is ubiquitous part of their daily diet, should improve pre- and post-harvest practices to prevent mycotoxins contamination. Basic training to farmers and food manufacturers on drying could already be a driving step to reduce the contamination levels. Removing the mouldy chillies and using good quality chilli pods for processing could be another simple option to prevent contamination. A watchful control by the responsible agencies over chilli traders is essential to avoid trade frauds and to ensure good quality products in the market. The study needs authoritative attention because this is the first study from Sri Lanka where consumption of chillies is substantially high and high contamination of aflatoxins and OTA were found. The information presented can be helpful for Sri Lankan agencies to take necessary actions during importation and to ensure safer food processing locally. The outcome of this single spice analysis already indicates the necessity of mycotoxins analysis in other Sri Lankan food products and the assessment of risk associated with combined mycotoxin exposure. Though evaluating the combined toxicity is foreseen highly complex, novel strategies need to be developed in order to perform toxicological research on the "cocktail effect" of multiple mycotoxins.

The mycotoxin contamination data of Sri Lankan chillies presented in this chapter was integrated with the chilli consumption data and a quantitative risk assessment was performed for the Sri Lankan context as described in the next chapter **(Chapter 5)**.

CHAPTER 5

PUBLIC HEALTH RISK ASSOCIATED WITH THE CO-OCCURRENCE OF MYCOTOXINS IN SPICES CONSUMED IN SRI LANKA

CHAPTER 5: PUBLIC HEALTH RISK ASSOCIATED WITH THE CO-OCCURRENCE OF MYCOTOXINS IN SPICES CONSUMED IN SRI LANKA

Summary

A quantitative risk assessment of mycotoxins due to the consumption of chilli (*Capsicum annum* L.) and black pepper (*Piper nigrum* L.) was performed in Sri Lanka. Mycotoxin concentration data used in this risk assessment study was the same as reported in the previous chapters; pepper in **Chapter 3** and chilli in **Chapter 4**. A food frequency questionnaire was administered in order to collect the data on consumption of spices by households in the Northern and Southern region (n=249). The mean chilli consumption in the North was significantly higher ($p<0.001$) compared to the South. The tolerable daily intake (TDI)/provisional TDIs (PTDI) established by JECFA or EFSA were used in the risk characterisation of different mycotoxins. Dietary exposure to, fumonisin B1 (FB1), fumonisin B2 (FB2), sterigmatocystin (STERIG) and citrinin (CIT) due to spices were estimated in addition to the most potent aflatoxin B1 (AFB1) and ochratoxin A (OTA). The probable daily intake of FB1 due to pepper or FB2 due to chilli consumption was far below the proposed PTDI of 2 µg/kg BW/day for fumonisins by JECFA. Comparing the CIT exposures with the proposed "level of no concern for nephrotoxicity" of 0.2 µg/kg BW/day, the results show that there is no risk (min-P99) associated with CIT exposure due to these spices consumption. Margin of exposure estimations (MoE) of STERIG shows no health concern due to both spices consumption. Mean exposure to aflatoxin B1 (AFB1) in the North (3.49 ng/kg BW/day) and South (2.13 ng/kg BW/day) have exceeded the tolerable daily intake due to chilli consumption at the lower bound scenario, while exposure to OTA was small. MoE estimations at the mean exposure to AFB1 were remarkably lower due to chilli (45-78) than for pepper (2315-10,857). Moreover, the hepato cellular carcinoma (HCC) risk associated with the mean AFB1 exposure through chilli at the lower bound was 0.046 and 0.028 HCC cases/year/100,000 based on the North and South consumption, respectively. AFB1 exposure via chilli should be considered as a great public health concern in Sri Lanka due to both high mycotoxin concentration and high consumption.

Keywords: *Mycotoxins, Spices, Sri Lanka, Margin of exposure, Risk assessment, Hepato Cellular Carcinoma*

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5.1. INTRODUCTION

Human beings are exposed to a wide range of chemicals, the uptake of which by the human body is mainly through food, water, air and dermal contact. In this regard, mycotoxins, are considered to be some of the most potent natural and unavoidable chemical contaminants humans are exposed to in their daily lives. In the last decade, several studies focusing on mycotoxins contamination in foodstuffs, mainly cereals, nuts and spices have been reported from many countries especially from Asia and Africa (Shephard et al., 2008a; Marín et al., 2013). Sri Lanka is a tropical nation in Asia where several highly valued spices are produced. The prevailing climatic condition in the island is very suitable for the cultivation of the spices but unfortunately it is also suitable for the mould infestations and thus contamination with mycotoxins.

Considering the wide range of toxicity of mycotoxins, the occurrence of mycotoxins in agricultural commodities has been recognized as a potential hazard for the human and animal health and warrants the need for an exposure assessment. Despite a wide range of publications on occurrence of mycotoxins in several food products in several countries, only limited data are available on mycotoxin exposure among different populations (Marín et al., 2013). There are considerable differences in mycotoxin occurrence between various regions of the world. There are also significant differences between countries and even within countries with regard to the intake of food commodities, thus making exposure assessments and therefore risk assessments country specific (Kuiper-Goodman, 1995). Compared to Africa and Europe, mycotoxin occurrence and subsequent risk assessment studies are rather limited in the Asian context. In China, only aflatoxin exposure was previously studied for spices (Zhao et al., 2013) and peanut (Ding et al., 2012), in Japan for several foods (Sugita-Konishi et al., 2010) and in Malaysia for nut and nut products (Leong et al., 2011). Nonetheless, there are no studies available on assessing the risk associated with multiple mycotoxins exposure in any of the other Asian populations.

Previous chapters have revealed that the spices are heavily contaminated with multiple mycotoxins. Therefore, the present study aims to evaluate the dietary risk associated with the intake of spices, chilli and black pepper contaminated with multiple mycotoxins in populations from the two regions in Sri Lanka using both deterministic as well as probabilistic approaches. Risk characterization of the genotoxic mycotoxins was carried out based on the margin of exposure approach using the bench mark dose lower confidence level (BMDL). Moreover, the population risk for hepato cellular carcinoma (HCC) attributable to the AFB1 intake was estimated.

5.2. MATERIALS AND METHODOLOGY

5.2.1. Collection of spice samples and analysis of mycotoxins

In total, 168 spice samples were collected from different regions of Sri Lanka during the period of 2011-2012. This included 86 chilli (*Capsicum annum* L.) and 82 black pepper (*Piper nigrum* L.) samples. Further details on sampling and the summary of analytical results of mycotoxin contamination used in dietary exposure estimations of black peppers and chilli can be found in **Chapter 3** and **Chapter 4**, respectively.

5.2.2. Collection of spice consumption data in Sri Lanka

A spice consumption survey was performed during the period of 2012-2013 in households from Northern and Southern regions. The ethnic groups of these regions have different culinary practices in the usage of spices. Hence, a food frequency questionnaire (FFQ) was prepared in order to collect the usual spice intake from these two regions. Beforehand, the developed questionnaire was pre-tested among few (n=20) households. The female head of the family was directly interviewed to gather the information on the amount of these spices used in curry preparations based on portion size pictures. Portion size pictures were prepared for chilli powder, black pepper powder and whole peppercorns. Actual weights of each spice portion sizes were recorded. Use of portion size pictures in food intake estimation has been validated in a previous study (Huybregts et al., 2008). Regarding the intake of whole chilli pods the participant was directly asked to provide the information on the number of chilli pods added in a curry per day. Participants have been requested to choose the appropriate portion size picture which could reflect the right amount of each spice added in each curry preparation. The amount of spices added was obtained separately for different curry preparations, meat, sea foods and vegetables and summed up to find the total spice consumed by the family (Fig. 5-1 and 5-2). Finally, the mean daily spice consumption of the individual was calculated based on the total number of family members (>15 years of age) assuming that the consumption of spices is equally shared among the family members. In total, 249 randomly selected households from Sri Lanka comprising 62 families (235 individuals) from the Northern region and 187 families (783 individuals) from the Southern region were interviewed, after obtaining their informed consent.

Assuming that the consumption of spices by children is insignificant, they were excluded (54 (13%) from North and 31 (7%) individuals from the South) from the calculations of the individual spice intake. Therefore, the consumption data reported in this study were from 181 individuals from the North and 752 individuals from the South. The gender distribution of the sample was 44% of male and 56% of female from the Northern and 48% of male and 52% of female from the Southern region. The ethical clearance was obtained from both Belgian (2012/082) and Sri Lankan (710/13) authorities in order to carry out this survey.

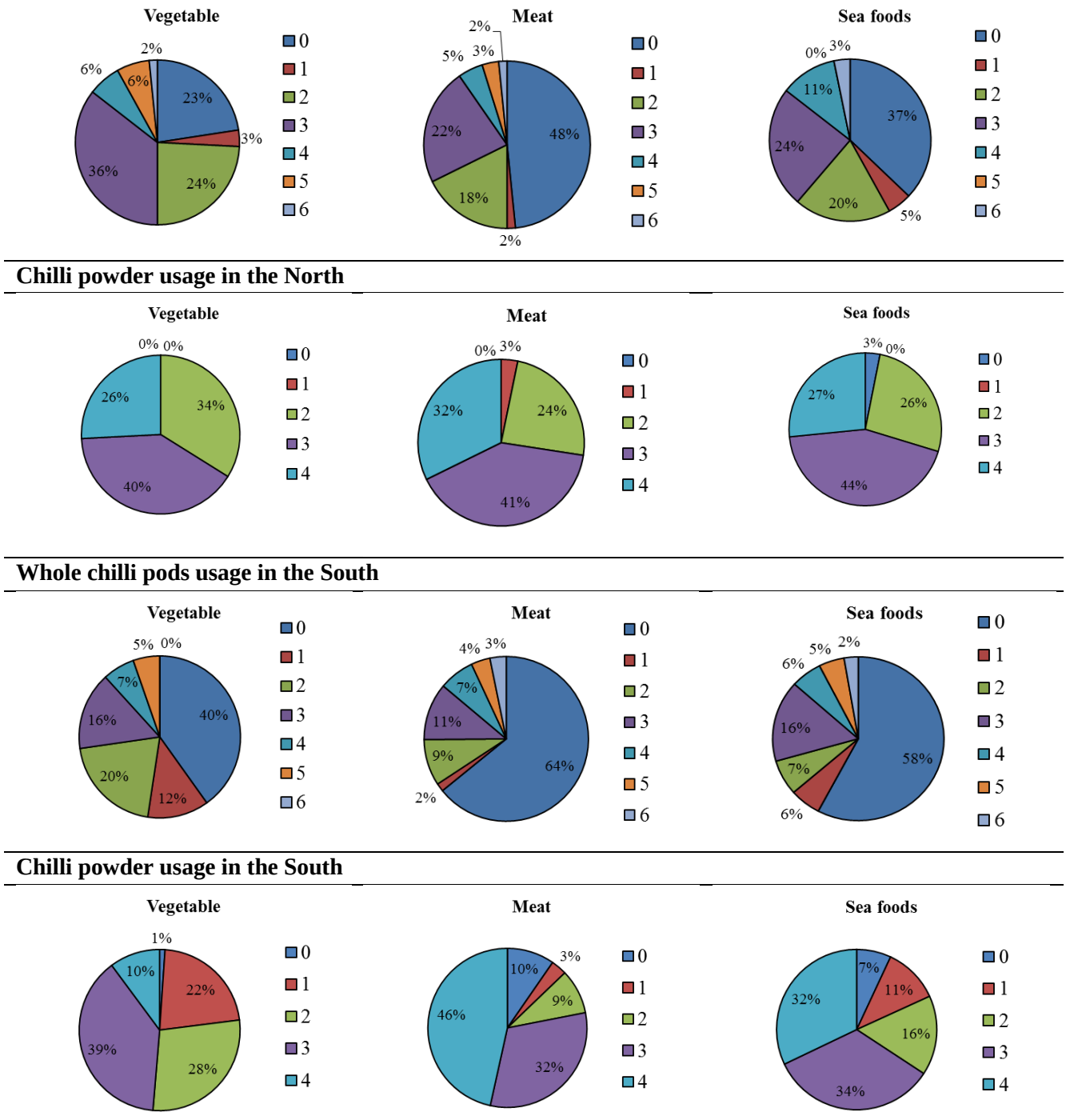
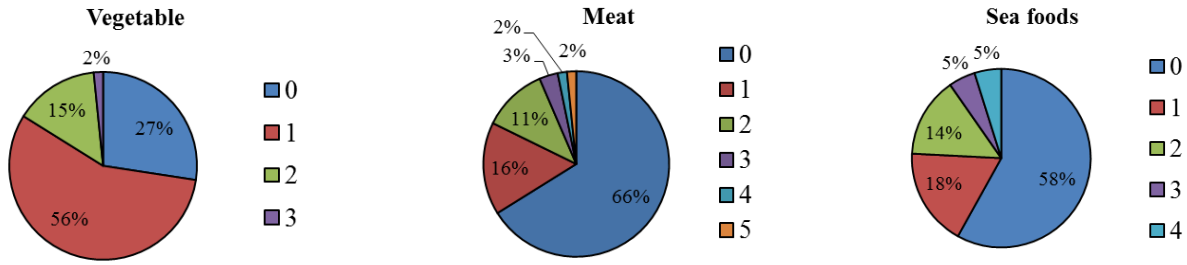
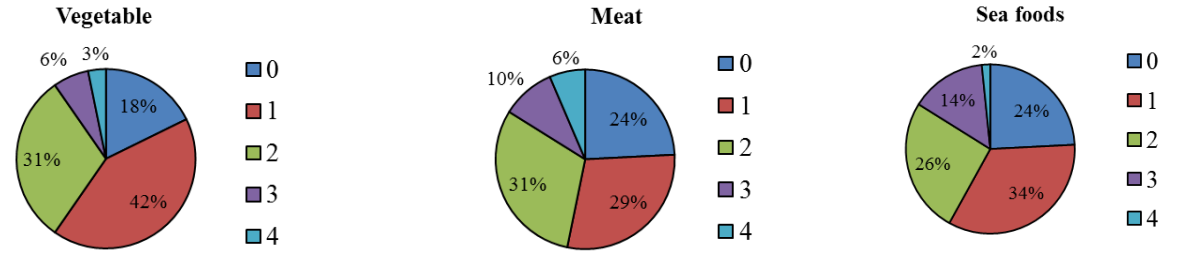


Fig. 5-1. Distribution of the percentage of families in the two regions of Sri Lanka and their usage of chilli powder and whole chilli pods in daily curry preparations (vegetable, meat and sea foods) based on the portion size pictures (legends denote the corresponding portion size, picture not shown; 1 is the smallest and 4 is the largest size). For whole chilli pods the legends denote number of chilli pods. Zero in any legend means chilli pod or chilli powder is either not added to any curry preparation or not reported.

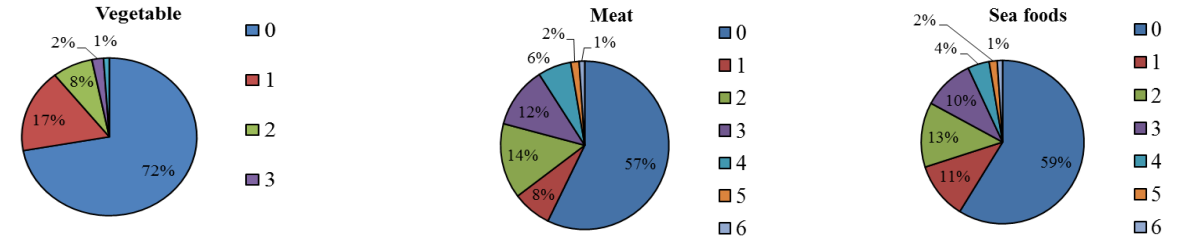
Whole black peppercorns usage in the North



Black Pepper powder usage in the North



Whole black peppercorns usage in the South



Black pepper powder usage in the South

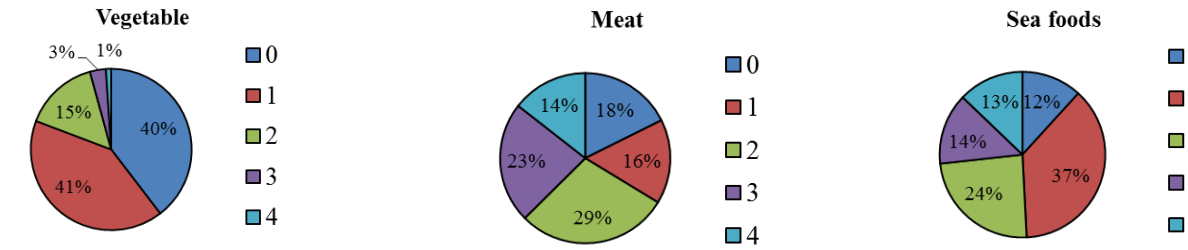


Fig. 5-2. Distribution of the percentage of families in the two regions of Sri Lanka and their usage of black pepper powder and whole black peppercorns in daily curry preparations (vegetable, meat and sea foods) based on the portion size pictures (legends denote the corresponding portion size, picture not shown; 1 is the smallest and 4 is the largest size). Zero in any legend means whole peppercorns or pepper powder is either not added to any curry preparation or not reported by the participant.

5.2.3. Exposure assessment

The most common approach to estimate the dietary exposure to mycotoxins is to integrate the contamination data obtained through the sample analysis and the consumption data generally obtained through national dietary surveys. Deterministic ("point estimations") and probabilistic ("simulated random sampling") methods were used to assess the risk associated with the mycotoxins exposure. However, some mycotoxins in spices had a low occurrence hence, no probabilistic approach could be conducted. Mycotoxin exposure assessment in this study incorporated the analysis of three different scenarios (lower, medium and upper bounds) related to the treatment of mycotoxin contamination data of the non-detects (NDs) and those below the limit of quantification (<LOQ) (Medeiros Vinci et al., 2012). Management of the left-censored contamination data is generally considered to be the main source of uncertainty in exposure models. Substitution of the NDs by the limit of detection (LOD) and zero for the upper bounds and lower bounds has been the most common approach in mycotoxin risk assessment studies (EFSA, 2010). Therefore, NDs were replaced by zero, half of the LOD and LOD, while <LOQs were replaced by half of the LOD, LOD and LOQ for the lower (LB), medium (MB) and upper bounds (UB), respectively. The exposure assessment was performed separately for chilli and pepper using the consumption data obtained from the population of the two regions in Sri Lanka. The mean body weight (BW) (n=933) calculated (mean±SD 59.85±4.17 kg) based on the report of survey participants was used in the exposure calculations.

5.2.3.1. Deterministic exposure assessment

A deterministic exposure assessment was performed considering the above explained three concentration scenarios. The spice consumption data obtained from the population of particular region was multiplied by the mycotoxin concentrations to determine the exposure associated with the particular mycotoxin of that region. Two deterministic approaches were performed for both the spices which could also help to identify potential acute toxicity associated with mycotoxins exposure. The exposure levels were estimated based on both the fixed mean mycotoxin concentration and the fixed mean consumption data using the minimum (min), mean, maximum (max) and the percentiles (P90, P95, P97.5 and P99) of the other exposure component in all the three scenarios considered.

5.2.3.2. Probabilistic exposure assessment

In addition to the deterministic approach, a probabilistic exposure assessment was carried out which considers the variances and uncertainties associated with the mycotoxins exposure determinants. Firstly, the fractions of the non-detects, <LOQ and >LOQ were calculated. For the risk output calculations, the fractions of >LOQ, <LOD + >LOQ, <LOQ + >LOQ were utilized using an "if"

logical function of the MS excel for the LB, MB and UB, respectively. Best fit distributions were determined for the three scenarios of the mycotoxin concentration data using the Chi-square statistics. The probability/probability (P/P) and quantile/quantile (Q/Q) plots were also assessed in order to determine the best fit distribution for both spice consumption and mycotoxins concentration data. The type of best fit distribution selected for the LB was also applied to the MB and UB of the concentration data. First order Monte Carlo simulations were performed considering 50,000 iterations. The simulations were repeated three times to ensure that the values remained stable. The simulations were performed with the add-in @risk® for Microsoft Excel version 6.1 (Palisade Corporation, USA). Probabilistic analysis was performed only for AFB1, OTA, STERIG in pepper and for AFB1 and OTA in chilli due to the low occurrence data of other mycotoxins. The probable dietary intakes of the mycotoxins (mean, standard deviation (SD), maximum and the percentiles) through the consumption of each of the spice were determined in both the regions.

5.2.3.3. Risk characterization

The calculated exposure values were compared with the established TDIs/provisional TDIs (PTDIs)/Provisional Maximum TDIs (PMTDIs) in order to evaluate the risk of the exposure. Since AFB1 is a genotoxic carcinogen, exposure at any level is considered unsafe. However, despite its uncertainty a PMTDI of 1 ng/kg BW/day was estimated by Kuiper-Goodman (1998) for adults and for children without hepatitis B. The PTWIs of OTA set by the European Food Safety Authority (EFSA, 2006) and the Joint FAO/WHO Expert Committee on Food Additives (JECFA, 2007) were 120 and 100 ng/kg BW/day, respectively. Hence, a PTDI value of 17.1 (EFSA, 2006) and 14.3 (JECFA, 2007) ng/kg BW/day could be derived to evaluate the risk of daily exposure of OTA. Comparison of the OTA exposures with these derived PTDI values was also performed in other studies (Coronel et al., 2011; Casal et al., 2014). For CIT, a value of 0.2 µg/kg BW/day ("level of no concern for nephrotoxicity") has been used to assess the risk as proposed by EFSA (2012). Moreover, for the non-genotoxic fumonisins a PMTDI value of 2 µg/kg BW/day was allocated by JECFA for alone (FB1, FB2 and FB3) or in combination (JECFA, 2001; SCF, 2003).

Additionally, for genotoxic mycotoxins the risk characterization was performed using two approaches. For further insight into the possible Sri Lankan exposures, first the Bench Mark Dose Lower confidence limit (BMDL), was combined with the AFB1 exposure associated with chilli and pepper to estimate the Margin of Exposure (MoE). This was used to quantify the increased cancer risk due to mycotoxin exposure. AFB1 concentrations estimated in all the three scenarios were used for the MoE calculations for both the spice consumption. MoE was calculated from a chosen point of departure (PoD) on a dose-response curve (lower limit of the BMD estimate at 95% confidence) divided by the human dietary exposure estimate. The BMDL value developed by the scientific panels of EFSA (2005b & 2007) was adopted, as a PoD to calculate the MoE to AFB1; which is $BMDL_{10}$ of

170 ng/kg BW/day for 10% increased cancer risk based on the rodent data. Moreover, the $BMDL_{10}$ of 0.16 mg/kg BW/day proposed by EFSA (2013), has been used for the risk evaluation of STERIG since it has also been identified as genotoxic and carcinogenic mycotoxin.

Secondly, estimations of the AFB1 induced liver cancer (hepato cellular carcinoma) incidences based on the procedure described by Liu and Wu (2010) were performed to characterize the risk. The exact problem of aflatoxin related HCC cases in Sri Lanka is unknown. Therefore, aflatoxin exposure levels estimated in this study were further incorporated in a quantitative cancer risk assessment to evaluate the overall cancer risk in Sri Lanka due to the consumption of spices. To perform a quantitative cancer risk assessment for aflatoxin-induced HCC in both the regions, the AFB1 exposure estimates from the deterministic exposure assessment were used. Because of the synergistic impact of aflatoxins in inducing HCC (Williams et al., 2004; Wu and Santella, 2012), the assessment was performed for both populations with and without chronic HBV infection. Firstly, the mean cancer potency factors for aflatoxins were calculated based on the report of the World Health Organization (JECFA, 1998) using the HBV prevalence in Sri Lanka. In Hepatitis B surface Antigen positive (HBsAg (+)ive) individuals, the cancer potency factor of aflatoxin for a year was assumed to be 0.3 while for the negative (HBsAg (-)ive) individuals it was 0.01 cancers/ng aflatoxin/kg BW in a population of 100,000 (JECFA, 1998). To estimate the aflatoxin exposed HCC cases of these two populations, the AFB1 exposure estimates of the min, mean, max and the percentiles obtained through the deterministic approach, were multiplied by the calculated mean cancer potency. Finally, the total number of aflatoxin induced HCC cases due to the consumption of contaminated spices in North and South were estimated using the population statistics (>15 years of age).

5.2.4. Statistical Analysis

The Kolmogorov-Smirnov and Shapiro-Wilk test and the corresponding Q/Q plots were assessed to determine the normality of the consumption data distribution. Due to the non-normal distribution of the obtained data, non-parametric Mann-Whitney U test was applied to assess the significance. The statistical analyses were carried out using the SPSS statistical package (IBM®, Version 22). Level of confidence was 0.05 unless otherwise specified. The mean data were mostly stated together with standard deviation (SD).

5.3. RESULTS AND DISCUSSION

5.3.1. Consumption of spices in Sri Lanka

The details of the population group (based on age) that participated in the spice consumption survey from North and South regions are presented in Fig. 5-3. The population group consisted of children (<15 years; North 13% and South 6%), adolescents (15-18 years; North 6% and South 6%), adult group 1 (19-29 years; North 28% and South 36%), adult group 2 (30-65 years; North 46% and South 45%) and elderly (>65 years; North 7% and South 6%).

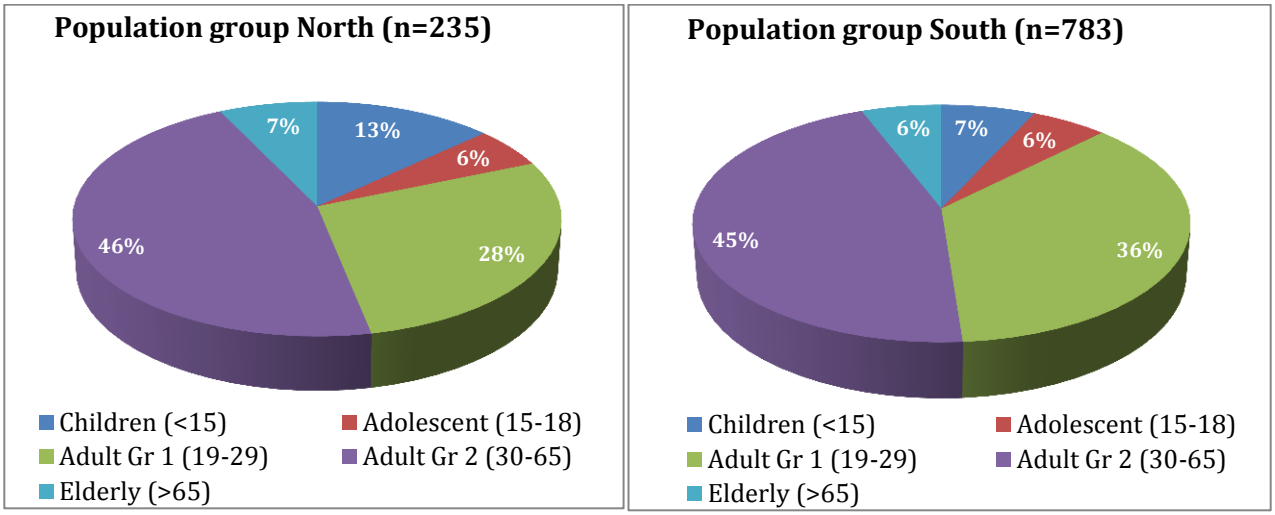


Fig. 5-3. The details of the population group (based on years of age) participated in the data collection on the consumption of spices chilli and black pepper, in Northern and Southern regions of Sri Lanka.

Based on this survey, the mean chilli consumption of the population from the Northern region (>15 years) was estimated to be 12.11±4.72 g/head/day while it was 7.38±4.31 g/head/day in the Southern region (Table 5-1). Similarly, mean black pepper consumption was estimated to be 1.72±1.28 and 1.12±1.08 g/head/day in the North and South, respectively. Total black pepper consumption included the consumption of whole black peppercorns and powder. A significant difference in chilli consumption between the two regions ($p<0.001$) was observed while pepper consumption between the two regions was not significantly different ($p=0.29$). On average, 9.74±4.52 g of chilli and 1.42±1.18 g of black pepper were consumed per capita on a daily basis. None of the households consumed white pepper. According to Pradeep et al. (1993), consumption of spices (including chilli and pepper) in India was 9.54±10.11 g/day, while it was 14.7 g/day in Thailand (Tantipopipat et al., 2010). These results are better comparable with our consumption results considering the fact that spices are widely used in Asian cuisine, while very low spice consumption (0.5 g/head/day) is reported in EU (Fowles et al., 2001).

Table 5-1. Consumption data (mean±SD g/head/day) of chilli and black pepper by the population above 15 years of age in Northern and Southern regions of Sri Lanka and at the national level (mean of North and South) obtained during the survey in 2012-2013.

Type of spice	Form of spice	Northern region	Southern region	National level
Chilli	Whole chilli pods	2.34±1.83 a*	1.34±1.53 a	1.84±1.68 a
	Chilli powder	9.77±4.41 b	6.04±3.69 b	7.90±4.05 b
	Total chilli	12.11±4.72 b	7.38±4.31 b	9.74± 4.52 b
Black pepper	Whole peppercorns	0.35± 0.47 a	0.18± 0.28 a	0.25± 0.38 a
	Pepper powder	1.37±1.14 b	0.95±0.99 b	1.16±1.07 b
	Total pepper	1.72±1.28 b	1.12±1.08 b	1.42±1.18 b

* Identical letters in each column denotes non-significant data for individual spice consumption.

5.3.2. Deterministic exposure assessment of the Sri Lankan population due to mycotoxins in spices

5.3.2.1. Chilli

Considering the exposure estimations from the Northern region, it is apparent that for AFB1 at LB (with the exception of the minimum level) all other levels exceeded the assigned PMTDI of 1 ng/kg BW/day (Table 5-2). The exposure estimations based on the two approaches (Table 5-2A and 5-2B) are almost similar until the percentile 97.5 (P97.5). However, it was observed that only at very high percentiles the exposure estimations are deviated highly which could be predictable due to the high AFB1 concentration in chilli (**Chapter 4**; Yogendrarajah et al., 2014b). Based on the literature review it was clear that across all the species the dose and duration of aflatoxin exposure clearly have a major effect on the toxicology and may cause a range of health consequences. Aflatoxicosis due to acute severe intoxication, results in direct liver cirrhosis and subsequent illness or death, while chronic sub-symptomatic exposure results in liver cancer (Williams et al., 2004).

Hence, in this comprehensive study two deterministic approaches have been used in exposure estimations of mycotoxins to explain all the possible chronic/acute exposures also by using the distribution of mycotoxin concentrations. For OTA, only at P99 and at the maximal level of the LB scenario does the exposure exceed the PTDI of 17.1 ng/kg BW/day (EFSA, 2006) (Table 5-2A). Considering the mean concentration approach (Table 5-2B) none of the exposures for OTA exceeds the PTDI.

Table 5-2. Deterministic dietary exposures (ng/kg BW/day) associated with the consumption of chilli contaminated with multiple mycotoxins by the populations in the North and South of Sri Lanka using the lower bound scenario. Calculations are based on A. fixed mean chilli consumption and variable mycotoxins concentration and B. fixed mean mycotoxin concentration and variable chilli consumption of the two regions. Values exceeding the PMTDI for aflatoxins and PTDI for OTA are shown in bold.

Deterministic approach	Descriptive level	AFB2		AFB1		OTA		FB2		STERIG		CIT	
		North	South	North	South	North	South	North	South	North	South	North	South
A. Fixed mean chilli consumption	Min	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	Mean	0.11	0.07	**3.49**	**2.13**	1.92	1.17	1.11	0.68	0.55	0.34	7.27	4.43
	Median	0.00	0.00	**1.05**	0.64	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	P90	0.15	0.09	**4.92**	**3.00**	2.97	1.81	6.50	3.96	1.08	0.66	0.00	0.00
	P95	0.31	0.19	**6.33**	**3.85**	4.17	2.54	6.50	3.96	1.08	0.66	14.8	9.00
	P97.5	0.31	0.19	**9.14**	**5.57**	5.52	3.36	6.50	3.96	1.08	0.66	14.8	9.00
	P99	2.00	1.22	**36.5**	**22.3**	**40.0**	**24.4**	8.17	4.98	6.58	4.01	170.3	103.8
	Max	6.38	3.88	**138.9**	**84.6**	**57.1**	**34.8**	17.6	10.7	7.45	4.54	426.2	259.6
B. Fixed mean mycotoxin concentration	Min	0.01	0.00	0.42	0.00	0.23	0.00	0.13	0.00	0.07	0.00	0.88	0.00
	Mean	0.12	0.07	**3.66**	**2.17**	2.01	1.19	1.16	0.69	0.58	0.34	7.62	4.51
	Median	0.11	0.06	**3.41**	**1.98**	1.87	1.08	1.08	0.63	0.54	0.31	7.09	4.11
	P90	0.18	0.12	**5.47**	**3.78**	3.00	2.07	1.74	1.20	0.86	0.60	11.4	7.87
	P95	0.21	0.15	**6.36**	**4.61**	3.49	2.53	2.02	1.47	1.00	0.73	13.2	9.58
	P97.5	0.21	0.16	**6.55**	**4.81**	3.59	2.63	2.09	1.53	1.03	0.76	13.6	10.0
	P99	0.24	0.16	**7.50**	**5.05**	4.11	2.77	2.39	1.61	1.18	0.80	15.6	10.5
	Max	0.29	0.18	**8.97**	**5.53**	4.92	3.03	2.86	1.76	1.41	0.87	18.7	11.5

The Southern region had a significantly ($p<0.001$) lower consumption of chilli compared to the Northern region. Although exposure levels were quite low, the mean AFB1 exposure is 2.13 ng/kg BW/day which is still more than twice the PMTDI (Table 5-2). OTA exposure of the Southern population is high at P99 and almost the double of the PTDI at the maximum of the LB scenario. Acute exposure to OTA is known to be nephrotoxic (EFSA, 2006). Overall, OTA exposure due to the consumption of chillies in Sri Lanka was very small.

The probable daily intake of FB2 due to chilli consumption was far below the proposed PTDI of 2 µg/kg BW/day for fumonisins (JECFA, 2001). Comparing the CIT exposures with the proposed "level of no concern for nephrotoxicity" of 0.2 µg/kg BW/day, the results show that there is no risk (min-P99) associated with CIT exposure due to spice consumption (Table 5-2). Only, the consumption of the very highly contaminated chilli exceeds this value, which could be the rarest acute toxicity case. CIT is acutely nephrotoxic at relatively high doses and it may have implications in the Balkan Endemic Nephropathy (BEN) when present with other toxins like OTA (EFSA, 2012). Due to the absence of Health Based Guidance Value (HBGV) for STERIG, risk characterization of STERIG was performed using the MoE approach.

According to Wanigasuriya et al. (2008), the levels of OTA found in food commodities were below the recommended statutory ML and are unlikely to be a potential risk factor for nephropathy in the North Central Province of Sri Lanka. Later on, a screening study was performed to determine the presence of nephrotoxic mycotoxins in urine samples from patients with chronic kidney disease of uncertain etiology in the same region. The percentage detection of aflatoxins, ochratoxins and fumonisins in 31 patients were 61.3%, 93.5% and 19.4%, respectively and concluded a high OTA exposure in this region (Desalegn et al, 2011).

5.3.2.2. Black pepper

The deterministic exposure levels associated with the mycotoxins AFG2, AFG1, AFB2, AFB1, FB1, OTA, STERIG and CIT due to the consumption of black pepper are shown in Table 5-3. The low mycotoxins exposure in pepper is due to the low mycotoxins concentration data obtained and the very low consumption of pepper (Yogendrarajah et al., 2014a; **Chapter 3**). Considering the deterministic approach of using the mean mycotoxin concentration at the LB (Table 5-3), the exposure to AFs due to pepper consumption ranged from 0-0.06 ng/kg BW/day, which is far below the PMTDI. For OTA the mean exposure is 0.07 and 0.05 ng/kg BW/day for the populations in the North and South, respectively. None of the concentration boundaries even at maximum concentrations exceeded the TDI for any of the aflatoxins, OTA, CIT and/or FB1.

A combined intake of different mycotoxins at different concentration levels may lead to higher risk than their single intake as emphasized by Speijers and Speijers in 2004. Anyhow, exposure to the most prevalent and potent mycotoxins associated with the consumption of pepper is notably low. Hence, it can be concluded that the mycotoxin exposure associated with the consumption of black pepper was not a health concern in this sample.

5.3.3. Probabilistic exposure assessment of the Sri Lankan population due to mycotoxins in spices

5.3.3.1. Chilli

The best fit distributions using Chi-square and descriptive statistics obtained for both the consumption of spices and mycotoxins contamination at the LB scenario are shown in Table 5-4. Plots of chilli consumption data from the South used in probabilistic analysis are shown in Fig. 5-4. The calculated dietary exposures due to mycotoxins in chilli and pepper in the two regions are shown in Table 5-5. The mean AFB1 intake due to chilli consumption was 0.12 and 0.08 ng/kg BW/day at LB for the North and South, respectively. The respective values for the MB and UB were 0.18 and 0.11, 0.37 and 0.23 ng/kg BW/day. These values are lower than the proposed PMTDI of AFB1. However, exposure above zero level is considered harmful for this genotoxic carcinogen (JECFA, 1998). Moreover, maximum exposures at the LB (0.92 ng/kg BW/day North, 0.84 ng/kg BW/day South) and MB (0.79 ng/kg BW/day North, 0.87 ng/kg BW/day South) should be of concern, since they are close to the PMTDI. The P99 of the UB (0.99 ng/kg BW/day) is almost equal to the TDI for the Northern population, while maximum exposure in both regions (1.82 ng/kg BW/day 82 in North and 1.45 ng/kg BW/day in South) exceed the TDI for AFB1.

Considering OTA, the mean dietary exposures at LB were 0.22, 0.11; for MB 0.68, 0.21 and for UB 0.67, 0.62 ng/kg BW/day, respectively for the Northern and Southern populations. These exposures and even at the higher percentiles were very well below the PTDI of OTA. Hence, it can be concluded that exposure to OTA due to chilli consumption is not a health concern in the studied populations in Sri Lanka.

Table 5-3. Deterministic dietary exposures (ng/kg BW/day) associated with the consumption of black pepper contaminated with multiple mycotoxins by the populations in the North and South of Sri Lanka using the lower bound scenario. Calculations are based on A. fixed mean pepper consumption and variable mycotoxins concentration and B. fixed mean mycotoxin concentration and variable pepper consumption of the two regions.

Deterministic approach	Descriptive level	AFG2		AFG1		AFB2		AFB1		FB1		OTA		STERIG		CIT	
		North	South	North	South	North	South	North	South	North	South	North	South	North	South	North	South
A. Fixed mean pepper consumption	Min	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	Mean	0.009	0.006	0.002	0.002	0.004	0.003	0.022	0.016	0.210	0.148	0.067	0.047	0.127	0.090	0.174	0.123
	Median	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	P90	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.348	0.246	0.000	0.000
	P95	0.059	0.042	0.000	0.000	0.055	0.039	0.139	0.098	0.893	0.630	0.178	0.125	0.446	0.315	2.114	1.491
	P97.5	0.155	0.109	0.000	0.000	0.055	0.039	0.303	0.214	3.521	2.484	0.497	0.351	0.622	0.439	2.380	1.679
	P99	0.222	0.156	0.035	0.025	0.055	0.039	0.432	0.305	3.675	2.592	1.831	1.292	1.041	0.734	2.530	1.784
	Max	0.234	0.165	0.183	0.129	0.055	0.039	0.480	0.339	3.730	2.631	2.192	1.546	1.358	0.958	3.108	2.192
B. Fixed mean mycotoxin concentration	Min	0.001	0.000	0.000	0.000	0.000	0.000	0.003	0.000	0.025	0.002	0.008	0.001	0.015	0.001	0.021	0.002
	Mean	0.009	0.006	0.002	0.002	0.004	0.003	0.022	0.016	0.210	0.148	0.067	0.047	0.127	0.090	0.174	0.123
	Median	0.009	0.005	0.002	0.001	0.004	0.002	0.022	0.012	0.210	0.113	0.067	0.036	0.127	0.069	0.174	0.094
	P90	0.016	0.014	0.004	0.003	0.007	0.006	0.038	0.034	0.363	0.325	0.116	0.104	0.220	0.197	0.301	0.270
	P95	0.021	0.015	0.005	0.004	0.009	0.007	0.051	0.038	0.483	0.359	0.155	0.115	0.293	0.218	0.401	0.298
	P97.5	0.022	0.018	0.006	0.005	0.010	0.008	0.055	0.046	0.521	0.432	0.167	0.139	0.316	0.263	0.432	0.359
	P99	0.023	0.021	0.006	0.005	0.010	0.010	0.058	0.053	0.544	0.496	0.174	0.159	0.330	0.301	0.451	0.412
	Max	0.025	0.022	0.006	0.006	0.011	0.010	0.061	0.055	0.579	0.522	0.186	0.167	0.352	0.317	0.480	0.433

Table 5-4. Best fit distributions and descriptive statistics (min, mean, median and max) for the lower bound scenario of the mycotoxin concentrations (µg/kg) and the consumption of spices (kg/kg BW/day) in both studied regions applied for the probabilistic exposure assessment.

Inputs of exposure assessment	Mycotoxins or Spice forms	Best fit distribution function	Min	Mean	Median	Max
Mycotoxin concentration in black pepper	AFB1	RiskExpon(6.18;RiskShift(1.25)	1.25	7.43	5.53	∞
	OTA	RiskExpon(19.94;RiskShift(2.38)	2.37	22.31	16.19	∞
	STERIG	Pearson5(1.67;6.27;RiskShift(7.28))	7.28	16.61	11.91	∞
Mycotoxin concentration in chilli	AFB1	RiskInvgauss(26.61;5.60;RiskShift(2.17)	2.17	28.78	10.47	∞
	OTA	RiskLoglogistic(4.65;7.53;1.53)	4.65	22.1	12.18	∞
Consumption of black pepper - North	Whole pepper corns	Expon(5.78E-06;RiskShift(-9.32E-08))	-9.3E-08	3.92E-06	5.69E-06	∞
	Pepper powder	RiskNormal(2.19E-05;1.59E-05)	−∞	2.2E-05	2.2E-05	∞
	Total pepper powder	RiskLogistic(2.65E-05;1.04E-05)	−∞	2.65E-05	2.65E-05	∞
Consumption of black pepper - South	Whole pepper corns	RiskExpon(3.18E-06;RiskShift(-1.72E-08)	-1.7E-08	3.16E-06	2.19E-06	∞
	Pepper powder	RiskInvgauss(2.02E-05;2.73E-05;RiskShift(-3.85E-06)	-3.9E-06	1.64E-05	1.11E-05	∞
	Total pepper powder	RiskBetaGeneral(0.10;3.48;2.85E-07;8.73E-05)	2.86E-07	1.97E-05	1.59E-05	8.73E-05
Consumption of chilli - North	Whole chilli pods	RiskExtvalue(2.59E-05;2.55E-05)	−∞	4.06E-05	3.52E-05	∞
	Chilli powder	RiskLoglogistic(-1.08E-04;2.6E-04;5.58)	-1.1E-04	1.72E-04	1.57E-04	∞
	Total chilli	RiskGamma(20.04;2.10E-05;RiskShift(-2.08E-04)	-2.1E-04	2.12E-04	2.05E-04	∞
Consumption of chilli - South	Whole chilli pods	RiskExtvalue(1.26E-05;1.78E-05)	−∞	2.28E-05	1.91E-05	∞
	Chilli powder	RiskExtvalue(7.74E-05;4.90E-05)	−∞	0.000106	9.53E-05	∞
	Total chilli	RiskExtvalue(9.61E-05;5.80E-05)	−∞	1.30E-04	1.17E-04	∞

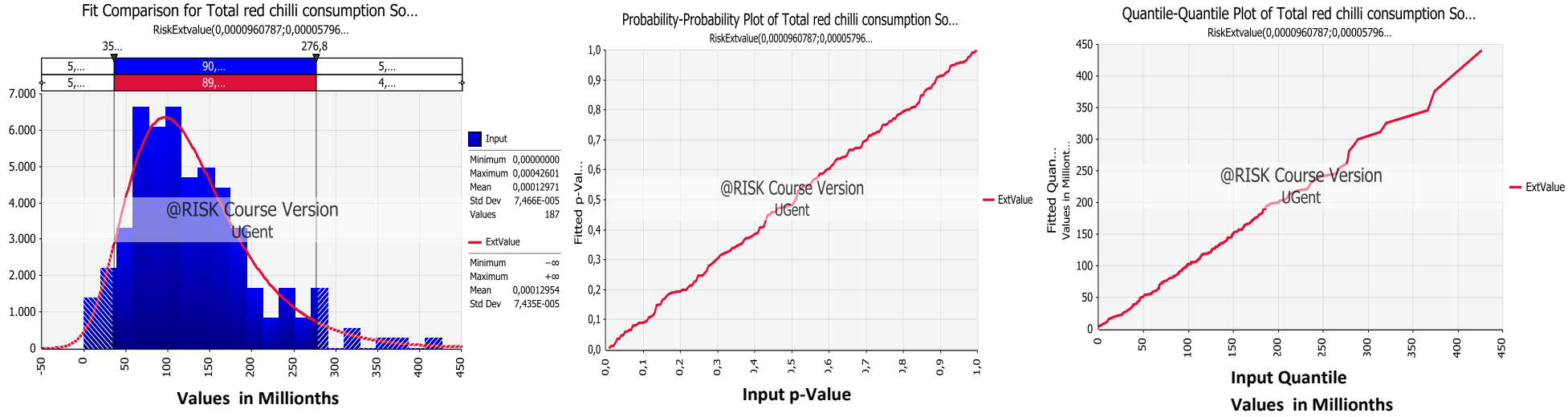


Fig. 5-4. Graphs on the best fit distribution of the total red chilli consumption from the Southern region of Sri Lanka and their Probability-Probability (P/P) plot and Quantile-Quantile (Q/Q) plots.

Table 5-5. Probabilistic dietary exposures (ng/kg BW/day) associated with the consumption of black pepper and chilli contaminated with mycotoxins AFB1, OTA and STERIG by the populations in the North and South of Sri Lanka using the lower bound and upper bound scenario of the mycotoxins concentrations. Values exceeding the PMTDI of AFB1 are shown in bold.

Mycotoxin concentration scenario	Descriptive level	Exposure due to chilli consumption				Exposure due to black pepper consumption					
		AFB1		OTA		AFB1		OTA		STERIG	
		North	South	North	South	North	South	North	South	North	South
Lower bound	Mean	0.12	0.08	0.22	0.11	0.03	0.02	0.09	0.06	0.05	0.04
	SD[a]	0.15	0.16	0.26	0.23	0.04	0.03	0.12	0.09	0.07	0.06
	Median	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	P90	0.33	0.21	0.61	0.31	0.08	0.07	0.26	0.21	0.16	0.12
	P95	0.39	0.26	0.71	0.38	0.10	0.09	0.32	0.27	0.19	0.16
	P97.5	0.44	0.31	0.80	0.45	0.12	0.10	0.37	0.32	0.22	0.19
	P99	0.49	0.37	0.90	0.55	0.13	0.12	0.43	0.38	0.26	0.23
	Max	0.92	0.84	1.64	1.25	0.25	0.17	0.79	0.53	0.63	0.32
Upper bound	Mean	0.37	0.23	0.67	0.62	0.08	0.06	0.34	0.25	0.15	0.11
	SD	0.21	0.33	0.38	0.88	0.07	0.05	0.24	0.20	0.12	0.10
	Median	0.32	0.19	0.59	0.55	0.07	0.04	0.34	0.20	0.14	0.08
	P90	0.67	0.43	1.21	1.09	0.17	0.13	0.63	0.54	0.31	0.25
	P95	0.78	0.53	1.42	1.31	0.20	0.17	0.73	0.65	0.38	0.33
	P97.5	0.88	0.62	1.59	1.51	0.23	0.20	0.82	0.73	0.44	0.39
	P99	0.99	0.75	1.80	1.78	0.27	0.24	0.95	0.82	0.51	0.45
	Max	**1.82**	**1.45**	3.14	3.54	0.52	0.32	1.78	1.07	0.97	0.62

[a] Standard Deviation

5.3.3.2. Black pepper

The probable dietary intake of AFB1 due to black pepper consumption was lower than the intake values obtained for the AFB1 in chillies (Table 5-5). The AFB1 intake values at LB were ranging from 0.03-0.25 and 0.02-0.17 ng/kg BW/day for the Northern and Southern populations, respectively. Also the intake estimates of other bounds were well below the PMTDI of AFB1. Furthermore, the dietary intake of OTA due to consumption of black pepper was 0.09-0.79, 0.06-0.53 ng/kg BW/day, for the North and South, respectively. Comparing both these exposure estimates with the PTDI, the current consumption level of pepper with the present OTA concentrations remains harmless. Moreover, the dietary intake of STERIG was assessed, since it was found in 43% of the Sri Lankan black peppers (15.4±9.8 µg/kg) (**Chapter 3**). The dietary exposure of STERIG ranged from 0.05-0.97, 0.04-0.62 ng/kg BW/day respectively in North and South considering all the scenarios of mycotoxin concentration. High contamination levels of STERIG in black peppers should not be neglected, warranting more occurrence data from several other food products and toxicity studies to establish a possible HBGV for STERIG. It should be noted that STERIG is the very last intermediate of the aflatoxin biosynthetic pathway and could express similar toxicity as AFB1 (Veršilovskis and De Saeger, 2010).

It is important to mention that a considerable difference on the exposure calculated based on both the deterministic and probabilistic approaches was obvious. This could be due to the higher variability in the mycotoxins concentration data than the consumption data. Moreover, a common limitation in mycotoxin exposure assessment studies is the large percentage of left-censored data from the occurrence studies and the heterogeneity of mycotoxin contamination in the samples (Marín et al., 2013). The exposure determinations at the high quartiles based on the deterministic approach using fixed consumption data might be useful in assessing the acute toxicity of AFB1 or combined toxicity with other mycotoxins. This could be particularly the case on a consumption of heavily contaminated spice; in our study two chilli samples had a very high AFB1 concentration, 91 and 687 µg/kg. Fatal acute AFB1 cases were previously reported in Kenya, India and Malaysia (Shephard, 2008b). Acute AFB1 toxicity is generally considered rare in developed countries, however when it occurs it could cause haemorrhagic necrosis of the liver, bile duct proliferation, oedema and lethargy (Williams et al., 2004).

The deterministic approach in this study provides a first insight on the exposure to multiple mycotoxins associated with spices in Sri Lanka. On the other hand, the probabilistic method is a powerful tool that allows us to know the validity and accuracy of the estimated high quartiles (Marín

et al., 2013). Yet, it could be best represented by the quality of the data distribution. In our study, the nature of the concentration data of some mycotoxins did not allow distribution fitting.

The assumption that spice intake by children is negligible (only considered population >15 years of age) and the limited sample size for consumption data collection could be the limitations of this study. Since, children are considered a vulnerable group for most contaminants, possible measures need to be identified in the future to determine their actual consumption. This could help to identify the relation of mycotoxin exposure on stunting or on immune system failures in children. Moreover, of the total population surveyed only 8% was children, thus it was far from representative to address this issue on children in this study. Nonetheless, we can conclude that the dietary exposures associated with the consumption of chilli are notably higher than that of black pepper and chilli should be considered as a spice of risk for public health. Hence, management strategies need to be focused more on the reduction of mycotoxin contamination in chillies.

5.3.4. Risk characterization due to spice consumption

5.3.4.1. Increased cancer risk based on the margin of exposure (MoE) approach

The calculated MoEs using the $BMDL_{10}$ of rodent data for the two spices in different regions for the three concentration scenarios are shown in Table 5-6. The estimated MoEs for chilli based on the exposures of fixed AFB1 concentrations (different levels of consumption) were all well below 10,000. These values are comparable to the MoE values reported for AFB1 in almonds, hazelnuts and pistachios by the EU, where estimated values ranged from 88 to 483 (EFSA, 2007). The mean MoE values obtained in our study (45-74) are similar to those reported in Africa (0.2-121.4) (Shephard, 2008b). MoE values of 5 and 833 were previously reported by Benford et al., (2010), for high and low exposed Asian populations. Although this study is only for spices, our results are consistent with these reported values as well. The mean MoEs due to probabilistic exposures for AFB1 were 1383±1174, 922±1607 and 461±804 for the North and 2255±1086, 1511±1032 and 754±515 for the South, at the LB, MB and UB, respectively (Fig. 5-5). The MoE values reported for AFB1 due to tree nut consumption in a Malaysian study ranged from 34 to 847 (Leong et al., 2011), while a MoE of 25-1273 was reported in a Chinese study for AFB1 in peanut (Ding et al., 2012).

According to EFSA (2005b), a MoE greater than 10,000 based on the $BMDL_{10}$ from rodent data could be considered as low health concern (Shephard, 2008b). In our case, the MoE values calculated for chilli are well below 10,000 hence, it should be considered as a public health concern in the studied households in Sri Lanka.

Table 5-6. Margin of exposure estimations using the rodent $BMDL_{10}$, based on the fixed mean aflatoxin B1 concentration and variable consumption data of pepper and chilli from the North and South of Sri Lanka.

Consumption level	Black pepper North			Black pepper South			Red chilli North			Red chilli South		
	LB	MB	UB	LB	MB	UB	LB	MB	UB	LB	MB	UB
Min	NA[a]	30,126[b]	19,476	743,605	347,657	224,758	403	400	393	NA[c]	NA	NA
Mean	7658	3580	2315	10,857	5076	3281	46	46	45	78	75	74
Median	7658	3580	2315	14,171	6625	4283	50	50	49	86	84	83
P90	4419	2066	1336	4937	2308	1492	31	31	30	45	42	41
P95	3321	1553	1004	4470	2090	1351	27	27	26	37	35	35
P97.5	3080	1440	931	3711	1735	1122	26	26	25	35	33	32
P99	2950	1379	892	3233	1512	977	23	22	22	34	27	26
Max	2770	1295	837	3076	1438	930	19	19	18	31	23	22

[a]Not applicable; MoE could not be calculated since the lower bound minimum is zero concentration.
[b]Margin of exposures with low public health concern (>10,000) are shown in shaded cells.
[c]Not applicable; zero consumption of chillies were reported in the South hence, there is no exposure.

Considering the black pepper intake the MoE values obtained were ranging from 930 to >>10,000 (zero exposure) in the deterministic scenario. In the North the MoE values for AFB1 in pepper were 7658, 3580 and 2315 for LB, MB and UB, respectively using the mean exposure estimations. Similarly, the MoE values for AFB1 in the South were 10,857, 5076 and 3281 (Table 5-6). Only the high percentiles of pepper consumption give rather low MoE values, indicating a potential health concern. Compared to the MoEs of chillies, the values obtained for pepper were very large (due to low exposure). This suggests that the health risk associated with the pepper consumption is comparatively low. However, only the Southern population under the LB scenario have the low risk according to the EFSA guidance value of low risk concern (>10,000) due to AFB1 in pepper. Moreover, the MoE (mean±SD) due to the probabilistic exposure of AFB1 via pepper in the North were 6336±4489, 4248±5191 and 2118±2594 for LB, MB and UB, respectively (Fig. 5-5). The respective values for the South were, 8647±5763, 5707±6386 and 2861±3182. Compared to the MoE values of chillies, the values for pepper were four to five times larger; meaning that they are of a lesser health concern. However, these exposures should not be undervalued since these MoE values are still below 10,000. Moreover, the magnitude of the MoE can be used by risk managers for priority setting and it provides more information than advising the exposures to be reduced to as low as reasonably achievable (EFSA, 2005b; Benford et al., 2010).

The MoE values for STERIG indicate no health concerns to occur due to the consumption of both chilli and pepper using both the probabilistic and deterministic dietary exposures (Table 5-7). Even at very high quartiles the MoE calculated using the deterministic approach, the MoEs were at least 2.1-3.9x10^4, which is higher than 10,000. The results indicate low risk due to STERIG exposure via consumption of these spices in Sri Lanka.

Table 5-7. Margin of exposure estimations using the STERIG $BMDL_{10}$ of 0.16 mg/kg BW/day based on the fixed mean STERIG concentration and variable consumption data of chilli and pepper from the North and South of Sri Lanka.

Concentration scenario	Consumption level	Chilli[a]		Pepper			
		Deterministic		Deterministic		Probabilistic	
		North	South	North	South	North	South
Lower bound	Min	NA[b]	NA	1.06E+07	1.22E+08	NA	NA
	Mean	2.91E+05	4.77E+05	1.26E+06	1.78E+06	3.15E+06	4.27E+06
	Median	NA	NA	1.26E+06	2.33E+06	NA	NA
	P90	1.48E+05	2.43E+05	7.26E+05	8.11E+05	1.02E+06	1.29E+06
	P95	1.48E+05	2.43E+05	5.45E+05	7.34E+05	8.48E+05	9.88E+05
	P97.5	1.48E+05	2.43E+05	5.06E+05	6.09E+05	7.39E+05	8.34E+05
	P99	2.43E+04	3.99E+04	4.84E+05	5.31E+05	6.28E+05	7.09E+05
	Max	2.15E+04	3.53E+04	4.55E+05	5.05E+05	2.56E+05	5.05E+05
Medium bound	Min	2.96E+05	4.86E+05	8.54E+06	9.86E+07	NA	2.92E+08
	Mean	1.81E+05	2.97E+05	1.02E+06	1.44E+06	1.58E+06	2.84E+06
	Median	2.96E+05	4.86E+05	1.02E+06	1.88E+06	1.58E+06	3.81E+06
	P90	1.48E+05	2.43E+05	5.86E+05	6.54E+05	8.52E+05	1.27E+06
	P95	1.48E+05	2.43E+05	4.40E+05	5.93E+05	7.37E+05	9.94E+05
	P97.5	1.48E+05	2.43E+05	4.08E+05	4.92E+05	7.40E+05	8.31E+05
	P99	2.43E+04	3.99E+04	3.91E+05	4.29E+05	5.67E+05	7.10E+05
	Max	2.15E+04	3.53E+04	3.67E+05	4.08E+05	2.79E+05	5.05E+05
Upper bound	Min	1.48E+05	2.43E+05	6.54E+06	7.55E+07	NA	1.47E+08
	Mean	9.96E+04	1.64E+05	7.77E+05	1.10E+06	1.06E+06	1.43E+06
	Median	1.48E+05	2.43E+05	7.77E+05	1.44E+06	1.19E+06	1.93E+06
	P90	7.40E+04	1.22E+05	4.49E+05	5.01E+05	5.10E+05	6.36E+05
	P95	7.40E+04	1.22E+05	3.37E+05	4.54E+05	4.26E+05	4.92E+05
	P97.5	7.40E+04	1.22E+05	3.13E+05	3.77E+05	3.67E+05	4.13E+05
	P99	2.43E+04	3.99E+04	2.99E+05	3.28E+05	3.14E+05	3.55E+05
	Max	2.15E+04	3.53E+04	2.81E+05	3.12E+05	1.65E+05	2.59E+05

[a] Probabilistic exposure assessment was not performed due to very small occurrence data of STERIG in chilli; most values were less than LOQ. [b] Not applicable; zero exposure.

5.3.4.2. Incidence of liver cancer/HCC due to AFB1 contamination in spices

AFB1, the potent human carcinogen is linked to human primary liver cancer in which it acts synergistically, highlighting a heavy cancer burden in developing countries or poor nations (Groopman et al., 2008; Liu and Wu, 2010; Wu and Santella, 2012). It is the most abundant of the aflatoxin analogues and considered as the most biologically active.

According to Groopman et al. (2008), the risk of liver cancer in individuals exposed to chronic hepatocellular B virus (HBV) infection and aflatoxins is 30 times higher than the risk in individuals exposed to aflatoxins only. The burden of aflatoxin induced HCC in different countries has been recently evaluated (Liu and Wu, 2010).

Chronic HBV prevalence in Sri Lanka is 0.2-1.9% (Andre, 2000). Thus, the mean cancer potency for the HBsAg (+)ive population in Sri Lanka could be calculated as 0.001-0.006 cancers per year per 100,000/ng AFB1/kg BW/day. The total population risk for the development of HCC in Sri Lanka was calculated to be 0.013 cancers/year/100,000/ng AFB1/kg BW/day. Taking into account the chronic HBV prevalence in SL, the risk estimates of aflatoxin induced HCC cases due to the synergistic impact with HBsAg (+)ive versus HBsAg (-)ive individuals for the deterministic scenario using fixed mean AFB1 concentration and variable consumption are shown in Table 5-8. Estimations of HCC cases were given for the populations >15 years of age using the AFB1 concentrations at MB using the latest population statistics (Central Intelligence Agency, 2013).

The liver cancer risk associated with the mean exposure at LB was estimated to be 0.046 and 0.028 cases/year/100,000 in the North and South, respectively. A Chinese study estimated 0.003-0.17 cancer cases/year/100,000 due to AFB1 in peanuts (Ding et al., 2012) in which a very high HBV prevalence rate (14.3%) was used. The estimated mean HCC incidences using the mean exposures are 7.8 and 4.6 of the total population considering the consumption of North and South, respectively (Table 5-8). Comparing the total HCC cases between the two regions, the cases in the North are slightly higher (1-20) than in the South (0-16) which is attributable to the high AFB1 exposure in the Northern region. The liver cancer risk associated with the black pepper consumption was 0.0003 and 0.0002 HCC cases/year/100,000 in the North and South, respectively. The results show that the incidences of HCC cases attributable to AFB1 in pepper were 0.01-0.07 in HBsAg (+)ive and 0.01-0.21 in HBsAg (-)ive individuals in the North and the respective values based on South consumption were 0.00-0.06 and 0.00-0.19 (Table 5-8).

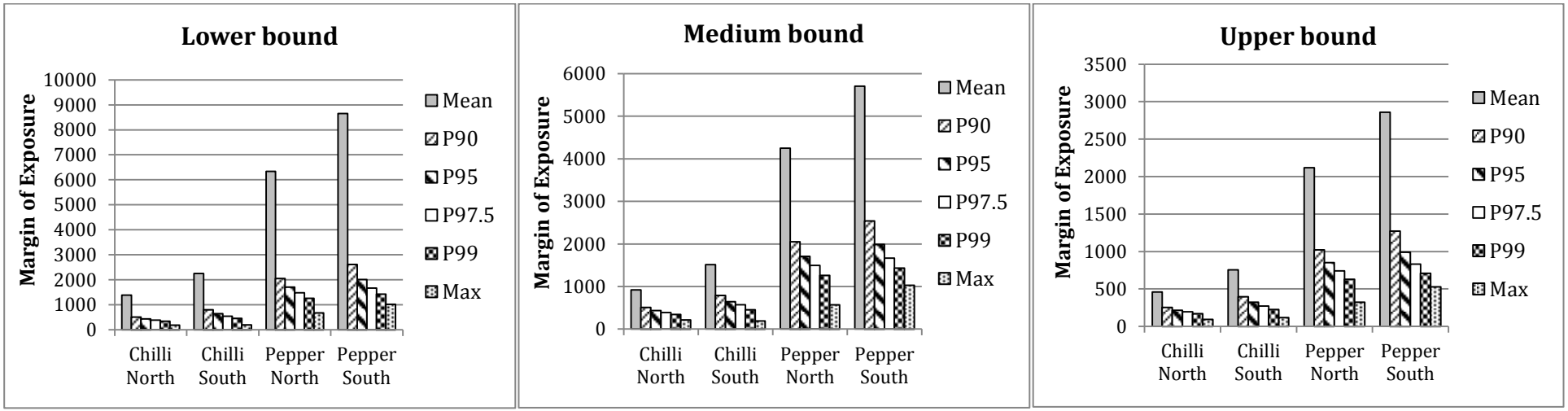


Fig. 5-5. Margin of exposures using the probabilistic exposure values at mean, maximum and percentiles of the three aflatoxin B1 concentration scenarios (lower bound, medium bound and upper bound) due to the consumption of chilli and black pepper by the populations in North and South of Sri Lanka.

Considering the mean AFB1 exposure the estimated mean liver cancer incidences were 0.10 and 0.07 in the North and South, respectively, which shows that there is no notable difference between the two regions. The total HCC cases in the North (0-0.28) and South (0-0.25) due to pepper consumption were comparable. Estimations of HCC cases due to peppers are very small compared to the HCC risk associated with AFB1 in chillies.

Table 5-8. Estimated annual burden of hepato cellular carcinoma (HCC) cases due to AFB1 exposure by chilli and pepper consumption in HBsAg-negative (HBsAg (-)ive) and HBsAg-positive (HBsAg (+)ive) populations in Sri Lanka based on the chilli consumption pattern in the Northern and Southern region. The exposures due to mean AFB1 concentration and variable consumption were used in estimating the HCC cases above 15 years of age.

Population	Consumption level	Chilli			Black Pepper		
		HBsAg (-)ive	HBsAg (+)ive	Total[a] HCC	HBsAg (-)ive	HBsAg (+)ive	Total HCC
North	Min	0.7	0.2	0.9	0.01	0.00	0.01
	Mean	6.0	1.9	7.8	0.08	0.02	0.10
	Median	5.5	1.7	7.3	0.08	0.02	0.10
	P90	8.9	2.8	11.7	0.13	0.04	0.17
	P95	10.3	3.3	13.6	0.18	0.06	0.23
	P97.5	10.7	3.4	14	0.19	0.06	0.25
	P99	12.2	3.9	16.1	0.20	0.06	0.26
	Max	14.6	4.6	19.2	0.21	0.07	0.28
South	Min	0.0	0.0	0.0	0.00	0.00	0.00
	Mean	3.6	1.2	4.8	0.05	0.02	0.07
	Median	3.2	1.0	4.3	0.04	0.01	0.05
	P90	6.6	2.1	8.7	0.12	0.04	0.16
	P95	7.8	2.5	10.2	0.13	0.04	0.17
	P97.5	8.3	2.7	11	0.16	0.05	0.21
	P99	10.3	3.3	13.6	0.18	0.06	0.24
	Max	12	3.8	15.8	0.19	0.06	0.25

[a]Total HCC cases were calculated for the population >15 years of age which is 75.2% of the total Sri Lankan population (16.3 million of the total 21.7 million) based on the Central Intelligence Agency (CIA) world fact book 2013.

Our results have shown that there is a potential risk in developing HCC due to AFB1 exposure via chillies. Based on these results it is obvious that among the two spices, chillies are of great public health concern due to both high mycotoxins concentration and high consumption. The HBV prevalence in Sri Lanka is quite low (0.2-1.9%) compared to many other Asian countries where rather high HBV prevalence (2-16%) is reported by Liu and Wu (2010). It is worth to mention that 85% of the liver cancer cases are from the developing world with high incidences in Eastern and South Eastern Asia and Middle and West Africa (Ferlay et al., 2010).

Adopting measures to reduce the aflatoxins contamination in food products and to improve the HBV vaccination programme are necessary to reduce the synergistic effect between HBV and aflatoxins. The risk characterizations of mycotoxins in individual spices presented in this study could be useful for the risk managers to directly set crop priority for adopting control measures. Some agricultural, dietary and clinical interventions to control the burden of aflatoxin exposure and to prevent the HCC cases worldwide are proposed (Wu and Khlangwist, 2010; Wild and Hall et al., 2000). Moreover, the extent of cancer burden will depend on the levels of cumulative exposure. The presented mycotoxin exposures of individual spices can be used in the future to estimate the global dietary exposure to mycotoxins in Sri Lanka. Moreover, it is important to evaluate the overall aflatoxin exposure through whole meal studies and to estimate the overall AFB1 induced HCC cases in Sri Lanka.

As mentioned before, only zero level of AFB1 exposure is allowed since it is a genotoxic carcinogen. Taking into account the heavy chilli consumption and high AFB1 contamination in chillies (67%>5 µg/kg; 44%>10 µg/kg; 12%>30 µg/kg), there is a necessity for a strict ML to be imposed in Sri Lanka. Even using an EU ML of 5 µg/kg at LB deterministic scenario, the MoE was calculated at 1053 which is still well below the 10,000 limit. Therefore, the ML for AFB1 in chilli should be even lower the 1 µg/kg at the current consumption level to keep the AFB1 exposure as low as possible (>10,000 MoE). Since, most of the dry chilli consumed in Sri Lanka is imported from India, the proposed ML could be useful for the Sri Lankan authorities, mainly to control the incoming consignments and later on to monitor locally during their extended storage and distribution.

5.4. CONCLUSIONS

This study reports the possible dietary exposure to multiple mycotoxins associated with the consumption of chilli and black pepper in Sri Lanka. The exposure to mycotoxins due to chilli was higher among the consumers from North region than those from South region due to higher chilli consumption. The margin of exposures associated with AFB1 was remarkably lower for chillies than that of peppers. Moreover, our results have indicated that there is a potential risk in developing aflatoxin induced HCC due to chilli consumption in Sri Lanka. Therefore, AFB1 exposure through chilli should be considered to be of high concern from a public health point of view. Moreover, characterizing the risk associated with the exposure to multiple mycotoxins continues to pose a major challenge in the field of risk assessment. Hence, warranting more toxicological studies on the "cocktail effect" of multiple mycotoxins exposure in human. Nevertheless, the data presented in this very first study of its kind are sufficient enough to indicate that there is a need for urgent attention by the risk managers to address the aflatoxin exposure due to chilli consumption in Sri Lanka.

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CHAPTER

6

MOISTURE SORPTION ISOTHERMS AND THERMODYNAMIC PROPERTIES OF WHOLE BLACK PEPPERCORNS (*Piper nigrum* L.)

CHAPTER 6: MOISTURE SORPTION ISOTHERMS AND THERMODYNAMIC PROPERTIES OF WHOLE BLACK PEPPERCORNS (*Piper nigrum* L.).

Summary

Adsorption and desorption isotherms for whole black peppercorns (*Piper nigrum* L.) were determined at 22, 30 and 37°C using the standard static gravimetric method. The sorption isotherms exhibited a type III behavior according to Brunauer-Emmett-Teller (BET) classifications. The equilibrium moisture content (EMC) decreased with increasing temperature at a particular a_w. Hysteresis existed over the entire a_w range at 30 and 37°C. But at 22°C, an intersection of the curves was found at a a_w ca. 0.75. Eleven commonly used sorption isotherm equations were fitted to the experimental sorption data. The goodness of fit of the models was evaluated using several statistical indices and residual plots. The Guggenheim-Anderson-de Boer (GAB) and Peleg models were determined to be the best models to describe the experimental sorption data. The Oswin, modified Oswin, modified Mizrahi, double log polynomial models were found to adequately describe the experimental data, whilst the Caurie, Smith, modified Henderson and the polynomial models were inadequate. The monolayer moisture content (M_0) (calculated using the GAB models) were 4.78, 3.56 and 3.49% at 22, 30 and 37°C for adsorption and 4.67, 4.67 and 4.36% for desorption, respectively. The maximum isosteric heat of sorption was 28.1 and 73.3 kJ/mol for adsorption and desorption, respectively, which was found at 4% EMC. The net isosteric heat of sorption and sorption entropy increased with an increase in EMC; after reaching the maximum at M_0, both curves dropped off exponentially. The Gibbs free energy change for adsorption ($-\Delta G$) ranged from 100.6-9370.6 J/mol depending on the temperature and EMC (1-40%). The study has provided useful information that could be applied during the industrial drying, processing and storage of whole black peppercorns. Based on our study, the black peppercorns can be stored at 22, 30 and 37°C, by reducing their moisture content (MC) to 10, 8 and 7%, respectively, with corresponding a_w values 0.57, 0.59 and 0.56. At 10% MC (as recommended by Spice Board of India) the a_w values were 0.67 and 0.70 at 30 and 37°C, respectively, which exceeds the minimum a_w limit for mould growth. Therefore, the safe storage limit of MC should be decided according to the storage temperature and this should be monitored along the supply chain.

Keywords: *Black peppercorns, Moisture isotherms, Adsorption, Desorption, Monolayer, Isosteric heat of sorption*

Relevant publication: Yogendrarajah, P., Samapundo, S., Devlieghere, F., De Saeger, S. and De Meulenaer, B. (2014). Moisture sorption isotherms and thermodynamic properties of whole black peppercorns (*Piper nigrum* L.). Submitted.

6.1. INTRODUCTION

Black pepper fetches the highest return as estimated from the volume of international trade. It is widely used around the world due to its pungent property and serves medicinal, preservation and insecticidal purposes (Srinivasan, 2007; **Chapter 1**). Similar to many other spices, they are mainly cultivated in tropical/semi-tropical countries and dried under the sun in open air. Apart from the climatic conditions, lack of Good Agricultural Practices (GAP) and Good Manufacturing Practices (GMP) are of great concern in developing countries where peppers are grown. Similar to many other dry foods, black peppers are susceptible to microbial spoilage and toxin production if their water activity (a_w) exceeds the safe limits and if storage temperature is suitable for microbial growth (Mandeel, 2005). They are often contaminated with several fungal species and more specifically with *Aspergillus* species which produce toxic secondary metabolites like aflatoxins and OTA (more information in **Chapter 3**).

The quality of most foods preserved by drying depends to a great extent upon their physical, chemical and microbiological stability. This stability is partly a consequence of the relationship between the equilibrium moisture content (EMC) of the food material and its a_w, at a given temperature. This relationship is described by moisture sorption isotherms. These isotherms are unique for individual food matrices and they are essential for modelling of drying processes, design and optimization of drying equipment, predicting shelf-life stability, calculating moisture changes which may occur during storage and in selecting appropriate packaging material (Gal, 1987). Since the quality of spices is markedly dependent on colour and flavour retention, it is essential to dry them to critical a_w values that preserve their quality by preventing oxidation of essential oils and enzymatic or microbial spoilage (Marcos et al., 1997). The determination of such critical values would allow the establishment of reasonable drying and storage limits for black pepper.

Numerous empirical and semi-empirical models are available that can be used to predict the moisture sorption properties of foods. These models can be divided into several categories; kinetic models based on a mono-layer (modified BET (Brunauer-Emmett-Teller) model), kinetic models based on a multi-layer and condensed film (Guggenheim-Anderson and de Boer (GAB) model), semi-empirical (Ferro-Fontan, Henderson and Halsey models) and empirical models (Smith and Oswin models) (Peng et al., 2007; Al-Muhtaseb et al., 2010). In the early 1980s, Van den Berg and Bruin collected and classified 77 such equations (Kiranoudis et al., 1993). Chirife and Iglesias (1978) found that the Halsey and Oswin models are also versatile. They reviewed 23 equations existing in literature for fitting moisture sorption isotherms of foods and food products. The best known two parameter homogenous model is the BET model but its applicability is limited to a_w values over the range of 0.1 to 0.45 (Al-Muhtaseb et al., 2002). The three parameter GAB model has been extensively used up to a_w of 0.9; above 0.9 it usually predicts less water content than experimentally measured. Both models

assume homogeneous sorption with water forming a mono-molecular layer initially, while the additional water forms a multilayer (Yanniotis and Blahovec, 2009). The monolayer moisture content (M_0) is of significant importance for the stability of food with regard to lipid oxidation, enzyme activity, non-enzymatic browning and structural characteristics. According to Lomauro et al. (1985) the GAB equation gave the best fit for more than 50% of the fruits, meats and vegetables than the two-parameter models. Three two-parameter equations and one three-parameter equation were evaluated for 163 food materials including fruits, vegetables, spices and starchy foods. A fourth parameter to the GAB equation was introduced by Viollaz and Rovedo (1999) in order to correlate the sorption data for a_w values higher than 0.9. Blahovec and Yanniotis (2008) introduced additional parameters to the GAB model assuming that the C constant of the GAB model is a function of a_w. Moreover, Peleg (1993) proposed a four parameter model that can be used for both sigmoidal and non-sigmoidal isotherms, and it performed as well as, or even better than the GAB model. The Langmuir model is one of the most well-known models and it has been extensively used in surface chemistry (Bretag et al., 2009).

Nonetheless, it has been already noted that no sorption isotherm model could fit data over the entire relative humidity (RH) range because water is associated with the food matrix by different mechanisms in different a_w regions. A detailed research of the literature showed that moisture sorption isotherms of foods can be described by more than one sorption model (Lomauro et al., 1985).

Although moisture sorption isotherm models have been developed for several food matrices, to date none have been developed for whole black peppercorns. Hence, the main objectives of the present study are to, a) determine the adsorption and desorption isotherms of black peppercorns at 22, 30 and 37°C, b) evaluate the ability of several commonly used sorption models to describe the experimental data and c) determine the thermodynamics of the sorption phenomenon.

6.2. MATERIALS AND METHODOLOGY

6.2.1. Materials

Whole black peppercorns (*Piper nigrum* L.) used in this study were of Sri Lankan origin purchased from a local market in Jaffna, Sri Lanka. The peppercorns had an initial moisture content of 12.5±0.7 g/100g dry matter and a_w of 0.60±0.06. Salts used for the development of moisture sorption isotherms were of analytical grade.

6.2.2. Experimental procedure for sorption isotherm development

Adsorption and desorption isotherms of the whole black peppercorns were determined at 22, 30 and 37°C, over a a_w range of 0.13-0.97 using the static gravimetric method. The equilibrium moisture

contents were determined at various equilibrium relative humidity values (ERHs, 13-97%) for both desorption and adsorption isotherms. For the adsorption isotherms the peppercorns were first dried in sealed jars over pure sulphuric acid (95%) until the weight loss was negligible. This took approximately one week and resulted in peppercorns with a a_w of 0.137±0.002. For the desorption isotherms, the peppers were first placed over water in sealed jars until no appreciable weight gain was noticed. This took *ca*. four weeks. Saturated salt solutions were prepared to achieve different ERHs; potassium acetate (23.1%), potassium carbonate (43.2%), magnesium nitrate (54.4%), sodium bromide (57.7%), potassium iodide (69.9%), sodium chloride (75.5%), ammonium sulfate (81.3%), potassium chloride (85.1%), potassium nitrate (94.6%) and potassium sulfate (97.6%). The ERHs given above are those achieved at 20°C.

The saturated salt solutions were placed in sealable plastic containers. Five grams of whole black peppercorns were placed in perforated aluminum cups and placed inside the containers with the saturated salt solutions. The cups were kept apart from coming into contact with the saturated salt solutions by means of a platform fashioned from chicken wire mesh. Four replicates were prepared for each condition (ERH and temperature) evaluated. Toluene (5 mL in an open container) was also placed inside each container to prevent fungal growth. The toluene level was regularly checked and refilled when necessary. The containers were then tightly closed and placed in the incubators at different temperatures (22, 30 and 37°C) for equilibration. The initial weight of the peppercorn samples together with the aluminum cups was recorded. The weight of the samples was recorded every week to check if the sample had equilibrated with the respective ERH. When there were no significant differences in weight the samples were removed from the incubator for measurement of the a_w and moisture content. The a_w values were measured using a water activity meter (Novasina LabMaster, Lachen SZ, Switzerland). The corresponding EMCs were determined by means of the oven drying method (AOAC, 1980).

6.2.3. Modelling of sorption isotherms

The equations shown in Table 6-1 were fitted to the experimental data. These sorption models are widely used for describing the sorption isotherms of food materials.

Table 6-1. The models applied to describe the adsorption and desorption experimental data of whole black peppercorns

Model	Equation[a]	Parameters	Eq. No
BET (Brunauer. Emmett and Teller, 1938)	$MC = \frac{M_0 C a_w}{[(1-a_w)+(C-1)(1-a_w)a_w)]}$	M_0, C	6-1
GAB (Guggenheim -Anderson and de Boer, 1966)	$MC = \frac{M_0 C K a_w}{[(1-Ka_w)(1-Ka_w+CKa_w))]}$	M_0, C, K	6-2
	$C = C_0 \exp(\Delta H_C / RT)$		6-3
	$K = K_0 \exp(\Delta H_k / RT)$		6-4
Peleg (Peleg, 1993)	$MC = k_1 {a_w}^{n_1} + k_2 {a_w}^{n_2}$	k_1, k_2, n_1, n_2	6-5
Oswin (Oswin, 1946)	$MC = k(a_w/1-a_w)^n$	k, n	6-6
Modified Oswin (Chen, 2000)	$MC = (A-BT)\left(\frac{a_w}{1-a_w}\right)^C$	A, B, C	6-7
Modified Mirzahi (Mizrahi and Karel, 1977)	$MC = \frac{a + a_w(ca_w+b)}{(a_w-1)}$	a, b, c	6-8
Caurie (Caurie, 1970)	$MC = \exp(1+ba_w)$	a, b	6-9
Smith (Smith, 1947)	$MC = a - bIn\,(1-a_w)$	a, b	6-10
Modified Henderson (Henderson, 1952)	$MC = \{In(1-a_w)/[-k(T+c)]\}^{1/n}$	k, c, n	6-11
Polynomial (Samapundo et al., 2007a)	$MC = a + ba_w + ca_w^2$	a, b, c	6-12
Double Log Polynomial (Bonner and Kenney, 2013)	$MC = b_3\,In(-ln(\mathrm{a_w}))^3 + b_2\,In(-ln(a_w))^2 + b_1 ln(-ln(a_w) + b_0$	b_3, b_2, b_1, b_0	6-13

[a] Moisture content is denoted by MC in all the equations

The goodness of fit of the model was evaluated by using the mean relative percentage deviation modulus (P) (see Eq. 6-14).

$$P(\%) = \frac{100}{n}\sum_{i=1}^{n}\frac{|\mathrm{EMC}_{exp,i} - \mathrm{EMC}_{pred,i}|}{\mathrm{EMC}_{exp,i}} \qquad \text{Eq. 6-14}$$

According to Lomauro et al. (1985), if the P value is less than 10% the model is considered to be acceptable.

Moreover, the accuracy of fit of the models was evaluated by calculating the root mean square error (RMSE) between the experimental and predicted EMCs (see Eq. 6-15).

$$\mathrm{RMSE} = \sqrt{\frac{1}{\mathrm{N}}\left[\sum_{i=1}^{N}(\mathrm{EMC}_{exp,i} - \mathrm{EMC}_{pred,i})^2\right]} \qquad \text{Eq. 6-15}$$

The suitability of the models was further evaluated by calculating the reduced chi square (χ^2) (see Eq. 6-16).

$$\chi 2 = \frac{\sum_{i=1}^{N}(\mathrm{EMC}_{exp,i}-\mathrm{EMC}_{pred,i})^2}{\mathrm{N}-\mathrm{n}} \quad \text{Eq. 6-16}$$

In Eq.'s. 6-14, 6-15 and 6-16, $EMC_{exp,i}$ is the i^{th} value of the experimentally measured EMC, $EMC_{pred,i}$ is the i^{th} predicted value of the EMC, N is the number of observations (data points) and n is the number of constants. A model was considered good when the R^2 was high and the RMSE, *P*(%) and χ^2 values were low.

Moreover, standardized residuals (residual sums of squares, see Eq. 6-17) were plotted as a function of the a_w values. A model is considered acceptable if the residual values are located in a horizontal band centered around zero, displaying no systematic tendencies (i.e. random in nature) towards a clear pattern. If the residual plot indicates clear pattern, the model is considered unacceptable or biased.

$$\mathrm{RSS} = \sum_{i=1}^{n}\left(\mathrm{EMC}_{exp,i} - \mathrm{EMC}_{pred.i}\right)^2 \quad \text{Eq. 6-17}$$

6.2.4. Thermodynamic properties of sorption phenomena

The thermodynamic properties of the sorption phenomena were evaluated in terms of net isosteric heat of sorption, Gibbs free energy, sorption entropy and binding energy in the whole black peppercorn-water system.

6.2.4.1. Determination of net isosteric heat of sorption

The net isosteric heat of sorption or enthalpy of sorption (q_{st}) is defined as the difference between the total heat of sorption (Q_{st}) and the heat of vaporization of water. The net isosteric heat of sorption is given by:

$$\mathrm{q_{st}} = \mathrm{Q_{st}} - \Delta \mathrm{H}vap \quad \text{Eq. 6-18}$$

The net isosteric heat of adsorption and desorption for a given moisture content was calculated using the Clausius-Clapeyron equation shown below:

$$\mathrm{q_{st}} = \frac{\mathrm{RT_1T_2}}{\mathrm{T_2}-\mathrm{T_1}} ln \frac{\mathrm{a_{w2}}}{\mathrm{a_{w1}}} \quad \text{Eq. 6-19}$$

where R is the universal gas constant (8.314 J/mol/K), a_{w2} and a_{w1} are the a_w values at absolute temperatures T_2 and T_1 (K), respectively. $\Delta Hvap$ is the heat of vaporisation (kJ/mol/K). qst was calculated from the slope developed by plotting $ln(a_w)$ versus 1/T for a specific moisture content. The slope equals $-q_{st}/R$ (Kiranoudis et al., 1993). The procedure was repeated for several EMCs to determine the relationship between the qst value and moisture content.

6.2.4.2. Determination of sorption entropy change

The sorption entropy change (ΔS) was determined using Eq. 6-20 (Aguerre et al., 1986).

$$-ln(a_w) = Q_{st}/(RT) - (\Delta S/R) \quad \text{Eq. 6-20}$$

The EMC data from the GAB model was used to determine the sorption entropy at each moisture content. The sorption entropy for a given moisture level was calculated using the intercept (ΔS/R) of the curve of $ln(a_w)$ versus 1/T.

6.2.4.3. Determination of Gibbs free energy change and binding energy

The change in Gibbs' free energy (ΔG) was calculated using the following equation,

$$\Delta G = -RT ln(a_w) \quad \text{Eq. 6-21}$$

To determine the free energy change due to change in the moisture content in whole black peppercorns, a_w data were generated by the GAB models for the sorption isotherms. Free energy changes were then computed at several EMCs for each temperature evaluated in this study.

The a_w data generated using the GAB models for the adsorption isotherms was used to calculate the binding energies as well. The equation derived by Brunauer et al. (1938), was used to calculate the binding energy (Kaleemullah and Kailappan, 2007).

$$C = K^{\left\{\frac{(\Delta H1 - \Delta H2)}{RT}\right\}} \quad \text{Eq. 6-22}$$

The binding energy ΔH_B can be replaced for ΔH1-ΔH2 in the above equation. Hence the equation can be rewritten as,

$$ln(C) = ln(K) + \left(\frac{\Delta H_B}{R}\right)\left(\frac{1}{T}\right) \quad \text{Eq. 6-23}$$

Using the linear Arrhenius plot of $ln(C)$ versus 1/T the slope ($\Delta H_B/R$) could be determined. The values of parameter C obtained from the BET models developed at the various temperatures were used in calculating the binding energy ΔH_B. The slope of the equation was multiplied by the universal gas constant (R) to obtain the binding energy during adsorption.

6.2.4.4. Determination of specific surface area of sorption

The specific surface area of sorption was determined using the following equation 6-24 (Arslan and Toğrul, 2006):

$$S = M_0 \times N_A \times A_m / M_{wat} = 35.3/M_0 \quad \text{Eq. 6-24}$$

where S is the solid surface area of sorption (m^2/g solids), M_0 the monolayer water content (%, dry weight basis), N_A the Avogadro's number (6.02E+23 molecules/mol), A_m the area of a water molecule (1.06E-19 m^2/molecule) and M_{wat} is the molecular weight of the water (18 g/mol).

6.2.5. Statistical Analysis

The statistical package SPSS (IBM®, Version 22) was used to estimate the parameters of the different models. Linear regression analysis was used to analyse the fit of the experimental data to the two parameter polynomial model whilst non-linear regression was used for the three and four parameter models. A one-way analysis of variance (ANOVA) was performed in order to identify the differences in EMCs between temperatures of both sorption isotherms.

6.3. RESULTS AND DISCUSSION

6.3.1. Desorption and adsorption moisture isotherms

The adsorption and desorption isotherms of whole black peppercorns developed at 22, 30 and 37°C are shown in Fig. 6-1. The EMCs of both the adsorption and desorption isotherms increased slowly between a_w values 0.2 and 0.75, followed by a steep rise at all the temperatures evaluated. Consequently, the sorption isotherms obtained in this investigation are type III (J shape) isotherms, which are characteristic of products holding small amounts of water at lower a_w values and higher amounts of water at high RH levels (Arslan and Toğrul, 2005). Similar behaviour has been reported previously in different food products i.e. sugars, apple, raisins, apricot, pineapple, beef (Al-Muhtaseb et al., 2002), pistachio powder (Yazdani et al., 2006) and Madeira cake (Al-Muhtaseb et al., 2002). A type III isotherm appears when the binding energy for the first layer is lower than the binding energy between water molecules (Al-Muhtaseb et al., 2002).

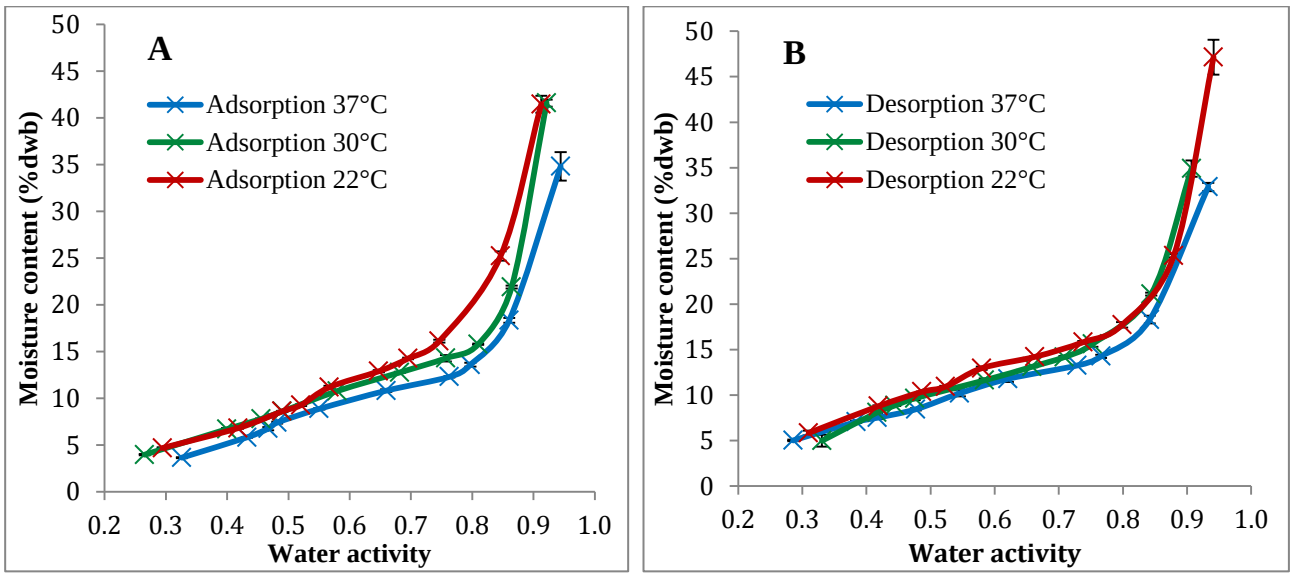

Fig. 6-1. Adsorption (A) and desorption (B) isotherms of whole black peppercorns at different temperatures.

The desorption curves lie slightly above the adsorption curve showing some hysteresis (Fig. 6-2). Hysteresis was observed over the entire a_w range at 30 and 37°C. However, at 22°C, an intersection of the curves was found at a_w *ca*. 0.75 (Fig. 6-2A). Crossing over of the sorption isotherms at high a_w (>0.90) was also reported in the study of texturized soy protein which further states that the cross

over a_w depends on the sugar content (Cassini et al., 2006; Tsami et al., 1990). Hysteresis has been related to the nature and state of the components in a food, reflecting their potential for structural and conformational rearrangements (Yan et al., 2008). According to a one-way ANOVA, the EMCs of both adsorption and desorption isotherms at different temperatures were found to be insignificantly different (p>0.05). Similar trends have also been observed in other foods including tea (Arslan and Toğrul, 2006), buffalo skim milk (Sawhney et al., 2013) and crushed chillies (Arslan and Toğrul, 2005). It is widely accepted that an increase in temperature results in a decreased moisture content at a particular RH. Temperature affects the mobility of water molecules and the dynamic equilibrium between the vapour and adsorbed phases. At the lower a_w values the adsorption isotherms at 22 and 30°C and desorption isotherms at all three incubation temperatures were found to overlap (Fig. 6-2).

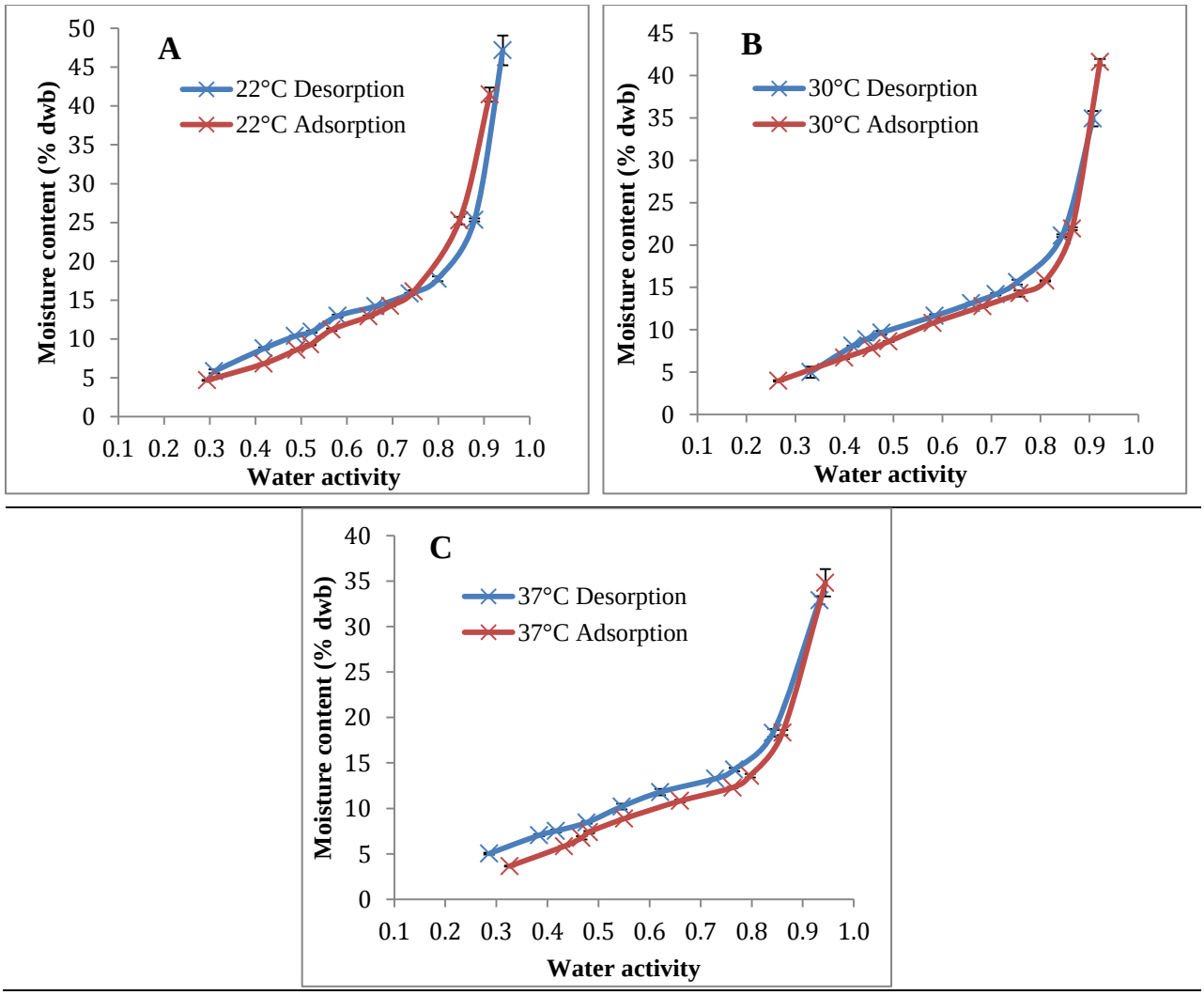

Fig. 6-2. Adsorption (—×—) and desorption (—×—) isotherms of whole black peppercorns at A) 22, B) 30 and C) 37°C showing the hysteresis.

The moisture sorption isotherms of grapes and apricot have also been determined to overlap at several temperatures (Kaymak-Ertekin & Gedik, 2004). The difference in EMC at the three incubation temperatures investigated in this study, became more clear in both the adsorption and desorption isotherms only at higher a_w values. According to Palipane & Driscoll (1992), an increase in temperature activates water molecules to higher energy levels, allowing them to break away from

their sorption sites, hence decreasing the EMC. As temperature varies, the excitation of molecules as well as the distance and hence, the attraction between molecules vary. This causes the amount of absorbed water to change with temperature at a given RH.

6.3.2. Fitting of sorption models to experimental data

As mentioned earlier, eleven models were fitted to the experimental adsorption and desorption isotherm data. The best/moderately and poor fitting models are shown in Fig. 6-3a and 6-3b, respectively. The estimated parameters and the statistical indices used to evaluate the goodness of fit of each model are shown in Tables 6-2 and 6-3.

Table 6-2. Estimated parameters and performance criteria of the best and moderately fitting models applied to the experimental adsorption and desorption data of whole black peppercorns.

Models and Parameters	**Adsorption**			**Desorption**		
GAB	**22°C**	**30°C**	**37°C**	**22°C**	**30°C**	**37°C**
M_0	4.78	3.56	3.49	4.67	4.67	4.36
C	11.9	12.1	12.7	9.07	4.65	6.61
K	0.97	0.99	0.95	0.95	0.95	0.93
R^2	1.00	0.97	0.99	0.97	0.97	0.96
R_{ave}	0.21	0.24	0.27	0.31	0.07	0.09
χ^2	0.61	7.60	6.17	3.92	2.35	2.74
P (%)	5.47	11.0	10.3	11.68	10.24	9.52
RSS	22.4	83.8	36.2	144.9	87.0	101.5
RMSE	0.75	1.67	0.95	1.90	1.48	1.59
PELEG						
k_1	80.8	168.4	51.5	92.5	66.5	41.7
k_2	22.3	19.4	17.3	21.5	19.9	15.8
n_1	14.5	23.7	17.6	20.5	14.6	11.7
n_2	1.31	1.15	1.24	1.03	1.05	0.83
R^2	1.00	1.00	1.00	1.00	0.98	0.97
R_{ave}	-0.01	-0.20	0.22	-0.01	-0.01	-0.01
χ^2	0.59	9.59	7.06	0.43	1.50	2.08
P (%)	3.42	4.67	6.78	2.99	6.04	6.09
RSS	21.1	249.2	254.2	15.4	54.0	74.7
RMSE	0.73	2.88	2.52	0.62	1.16	1.37
OSWIN						
K	8.36	6.97	6.74	9.19	8.91	8.39
n	0.67	0.70	0.58	0.57	0.57	0.52
R^2	1.00	0.96	0.99	0.97	0.97	0.96
R_{ave}	0.05	-0.12	0.26	0.20	0.07	0.05
χ^2	1.03	4.62	7.10	1.89	2.39	2.79
P (%)	4.89	15.3	10.6	10.3	9.46	7.92
RSS	39.0	129.3	269.7	135.3	90.9	106
RMSE	0.99	2.08	2.60	1.84	1.51	1.63
Modified OSWIN						
A	33662.8	204907.6	182019.9	-33660.3	-148653.2	256922.5

B	-1529.8	-6830.0	-4919.3	1530.4	4955.4	6944.1
C	0.65	0.70	0.52	0.57	0.57	0.52
R^2	1.00	0.96	0.92	0.97	0.97	0.96
R_{ave}	0.53	-0.10	-0.07	0.17	0.08	0.05
χ^2	1.87	4.76	6.54	3.66	2.46	2.87
P (%)	4.96	15.4	10.3	10.3	9.55	7.92
RSS	69.3	128.4	241.9	135.4	90.9	106
RMSE	1.32	2.07	2.46	1.84	1.51	1.63
Modified MIZRAHI	**22°C**	**30°C**	**37°C**	**22°C**	**30°C**	**37°C**
a	-1.12	-2.84	2.88	-4.06	-2.46	-2.02
b	-11.0	-5.02	-22.3	-5.71	-8.71	-9.84
c	9.12	5.30	18.5	7.58	8.81	10.3
R^2	0.99	0.95	0.90	0.99	0.97	0.96
R_{ave}	0.00	-0.37	0.00	-0.01	0.00	0.00
χ^2	0.67	1.87	7.66	1.36	2.02	2.85
P (%)	4.28	9.38	8.09	7.20	8.23	6.55
RSS	24.9	50.6	283.4	50.1	74.6	105.5
RMSE	0.79	1.30	2.67	1.12	1.37	1.62
Double Log Polynomial (DLP)						
b_0	6.25	6.42	5.35	7.66	6.66	6.78
b_1	-8.48	-7.13	-2.15	-10.8	-10.5	-5.52
b_2	-2.98	-3.60	3.94	-6.81	-6.11	-0.73
b_3	-2.32	-2.33	0.46	-2.83	-2.83	-0.89
R^2	1.00	0.95	0.93	1.00	0.98	0.96
R_{ave}	0.01	0.00	0.00	0.00	0.00	0.00
χ^2	0.62	5.71	5.87	0.56	1.61	2.64
P (%)	4.22	6.19	13.9	3.68	4.55	6.17
RSS	22.3	148.5	211.2	20.1	58.0	95.0
RMSE	0.75	2.23	2.30	0.71	1.20	1.54

The GAB model estimated M_0's of 4.78, 3.56 and 3.49% (dry weight basis, dwb) at 22, 30 and 37°C from the adsorption data. The desorption isotherms had higher M_0's of 4.67, 4.67 and 4.36% (dwb) at 22, 30 and 37°C, respectively. These results show that the M_0 decreases with increasing temperature (M_0 might depend on the models used). This could be due to the water molecules attaining the energy required to break away from their sorption sites at higher incubation temperatures, resulting in lower M_0's. The M_0 is a critical parameter as it represents the moisture content at which the rate of any associated reaction will be negligible due to the strong binding of water to the surface. An increase in a_w equivalent to an increase of M_0 by 0.1 units decreases the shelf life of a food product by a factor of 2-3 (Labuza, 1984). M_0 is therefore an important quality parameter with regard to the designing of optimal storage conditions for food products (Taitano et al., 2012). Therefore the M_0 values reported in this study represent the optimal moisture content for the storage of whole black peppercorns. However, it is practically unlikely to reach this low levels since they were very strongly bound with the matrix. Despite, it is very difficult to reduce the moisture content (MC) to this low level, highly reduced MC might affect the sensorial properties (quality) of the peppercorns.

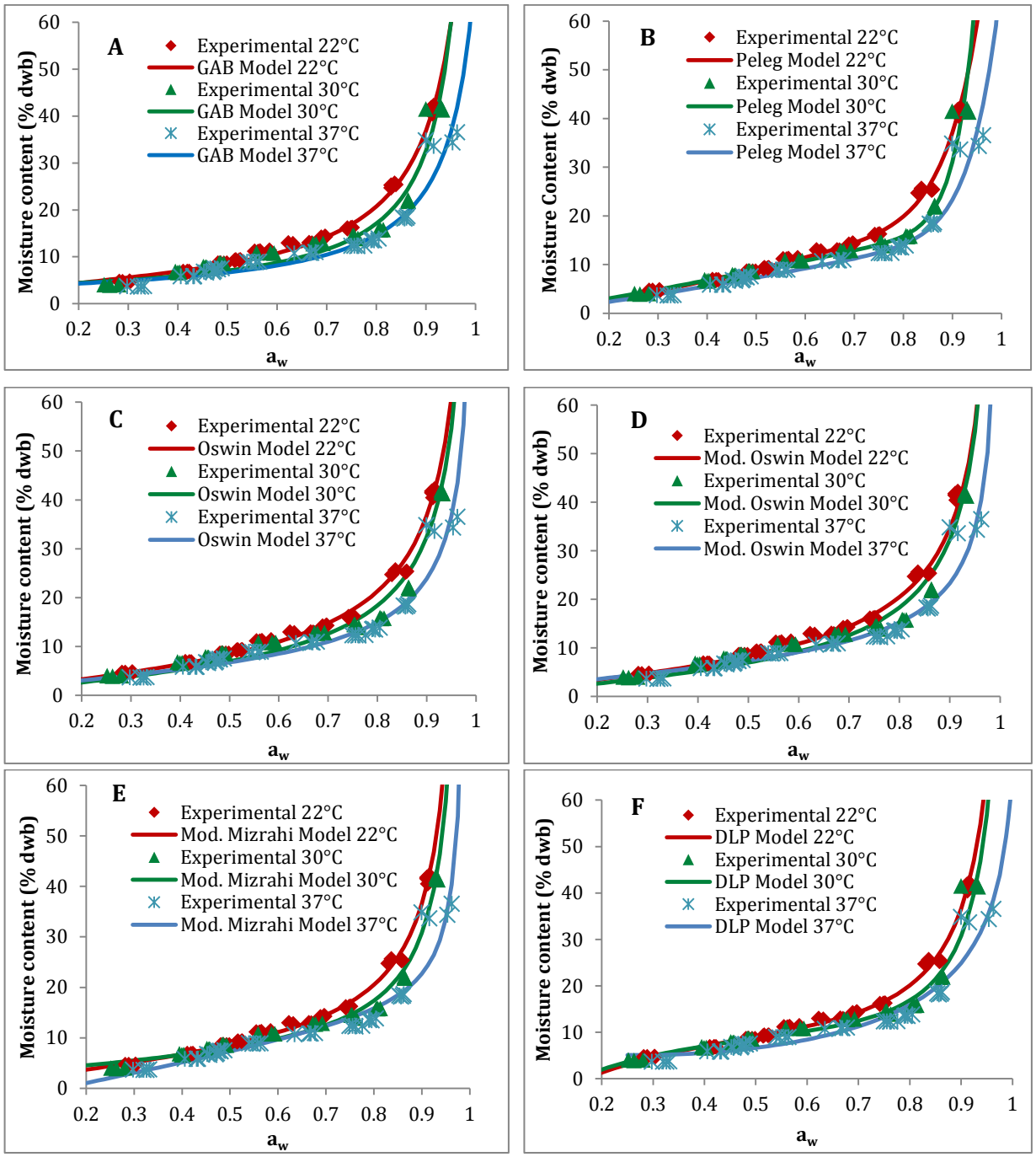

Fig. 6-3a. Mathematical models (best and moderate fitting A. GAB, B. Peleg, C. Oswin, D. modified Oswin, E. modified Mizrahi and F. double log polynomial (DLP)) fitted to the adsorption data of whole black peppercorns at different temperatures; experimental values are shown as markers (◆22°C,▲30°C and ✳37°C) and the model predictions are shown as lines (— 22°C, — 30°C and — 37°C).

The black peppercorns can be stored at 22, 30 and 37°C, by reducing their MC to 10, 8 and 7%, respectively, the corresponding a_w values were 0.57, 0.59 and 0.56 (based on the GAB model). At 10% MC as recommended by the Spice Board of India for safe storage for black peppers, the a_w values were 0.67 and 0.70 at 30 and 37°C, respectively, which exceeds the minimum a_w limit for mould growth. Therefore, the safe limit of MC (corresponding to 0.60 a_w) should be decided according to the storage temperature. Moreover, the a_w should be monitored throughout the supply chain inorder to ensure the safety and quality of the product.

As can be seen in Table 6-2, the estimated values of the GAB constant *C* (related to thermal effects) for adsorption were higher than those for desorption at all the temperatures evaluated. In agreement with the results from other products (Chirife et al., 1992), the estimated values for the constant *K* in the GAB model for both adsorption and desorption sorption isotherms were lower than unity. The physical meaning of *K* is related to the properties of the multilayer water molecules with respect to the bulk liquid. Moreover, the *C* values are much higher than *K* indicating that the heat of sorption of the first layer is greater than that of the multilayers.

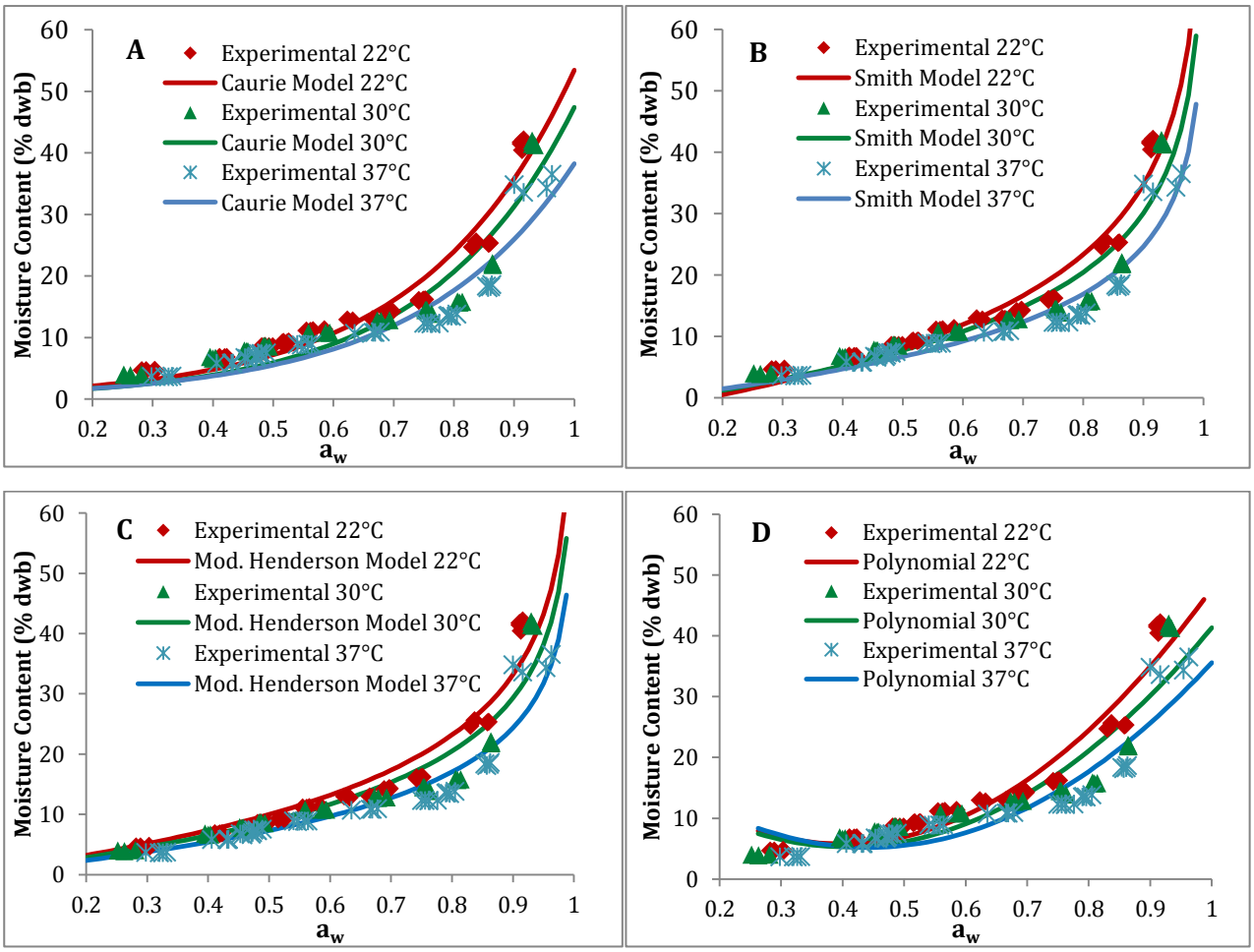

Fig. 6-3b. Mathematical models (poor fitting A. Caurie, B. Smith, C. modified Henderson and D. polynomial) fitted to the adsorption data of whole black peppercorns at different temperatures; experimental values are shown as markers (◆22°C,▲30°C,✳37°C) and the model predictions are shown as lines (—22°C, — 30°C and — 37°C).

As mentioned above, the goodness of fit of the models was assessed by means of the coefficient of determination R^2, *P*(%), reduced chi squared value (χ^2), residual sums of square (RSS), residual average (R_{ave}), root mean square error (RMSE) and the residual plots. Generally, a model with a *P* value of less than 10% is considered acceptable (Corrêa et al., 2007; Lomauro et al., 1985). According to the statistical indices, the model which fitted the experimental sorption data the best was the four parameter Peleg model which had R^2 values ≥ 0.98, the lowest *P* values (2.99-6.78%)

and residual averages (-0.01-0.02) at all the temperatures experimented. According to the residual plots (Fig. 6-4a), this model also shows no bias.

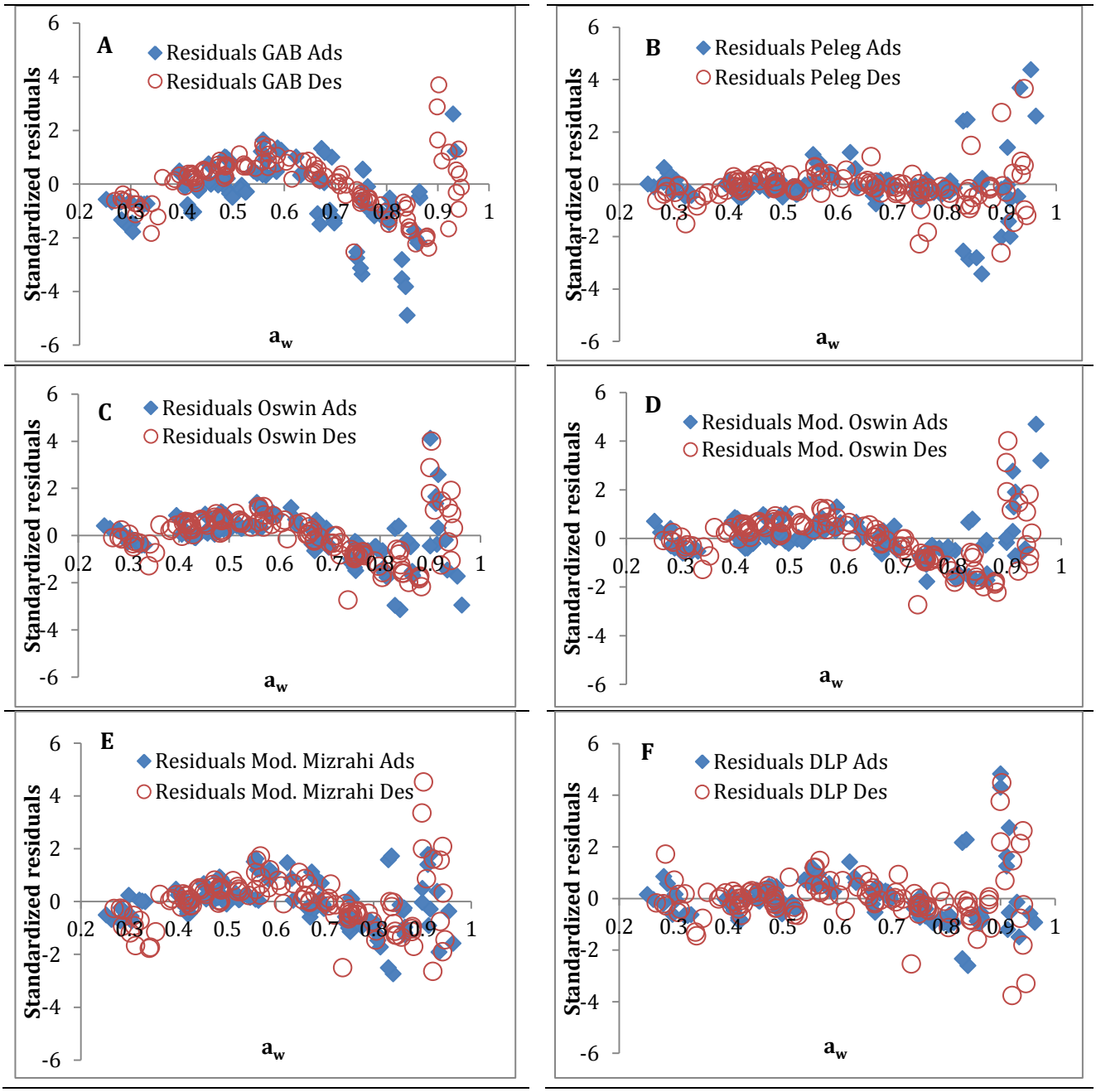

Fig. 6-4a. Residual plots (best and moderate fitting models A. GAB, B. Peleg, C. Oswin, D. modified Oswin, E. modified Mizrahi and F. double log polynomial (DLP)) of adsorption/Ads (◆) and desorption/Des (O) isotherms of whole black peppercorns fitted to different models.

The second best fitting model was the GAB model (*P* values 5.47-11.68%), for both adsorption and desorption curves ($R^2 \geq 0.96$). The residual plots show that in comparison to the Peleg model, the GAB model is slightly biased. The two parameter Oswin model and the modified Oswin model also had good fits to the experimental data with R^2 values ≥ 0.96 and *P* values ranging from 4.89 to 15.3%. However, as observed for the GAB model, the Oswin model is slightly biased in comparison to the Peleg model, albeit less than the GAB model. The modified Mizrahi model also had good *P* values ranging from 4.28 to 9.38%, for both adsorption and desorption isotherms. However, in

comparison to the excellent fit of the modified Mizrahi model on the desorption data, slightly lower R^2 values of 0.95 and 0.90 were obtained when the model was fitted to the adsorption isotherm data generated at 30 and 37°C, respectively.

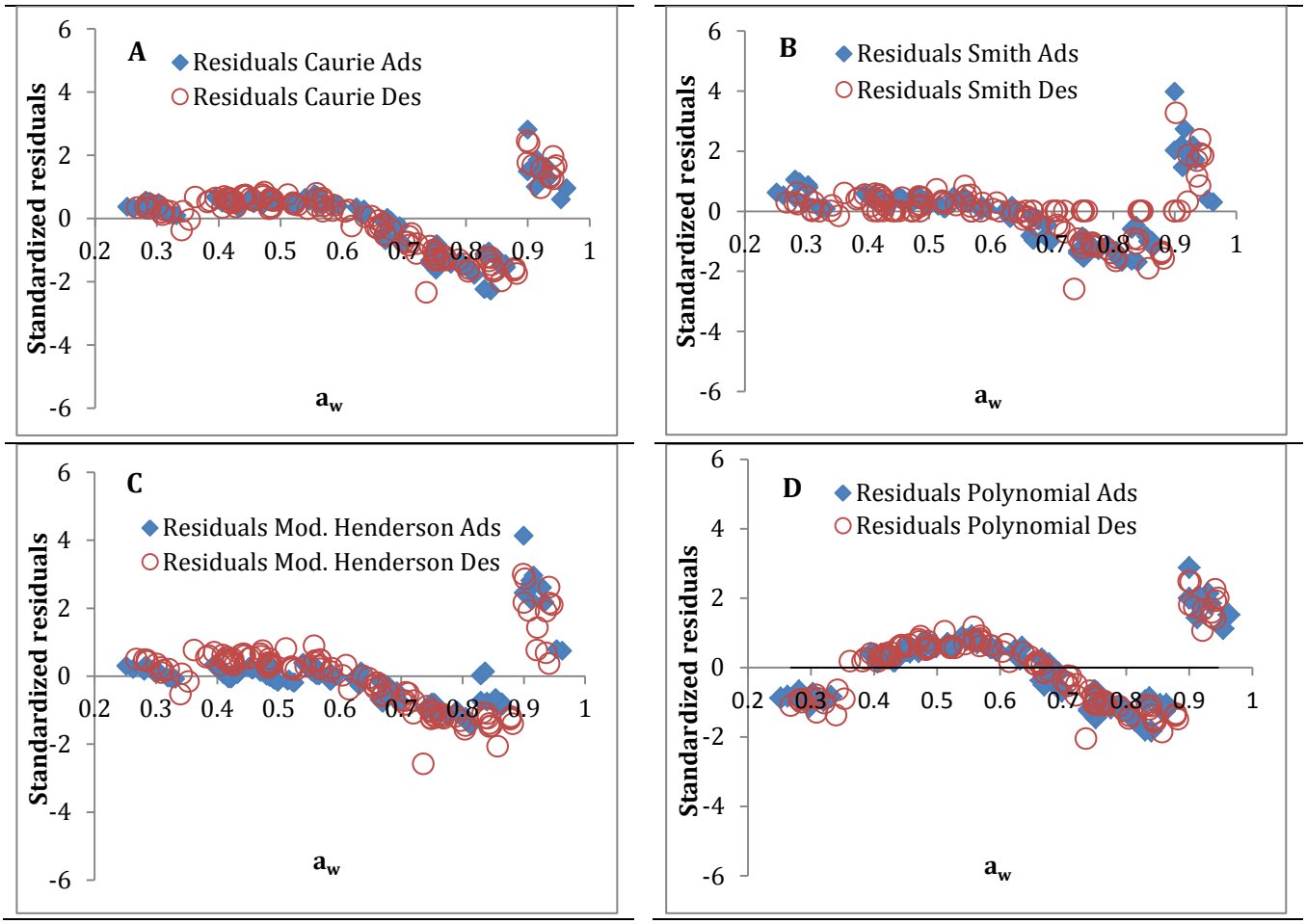

Fig. 6-4b. Residual plots (poor fitting models A. Caurie, B. Smith, C. modified Henderson and D. polynomial) of adsorption/Ads (◆) and desorption/Des (O) isotherms of whole black peppercorns fitted to different models

The double log polynomial (DLP) model was also determined to adequately fit the experimental sorption data with R^2 values ≥ 0.93 and *P* values ranging from 3.68 to 13.92%. The Caurie (R^2, 0.85-0.95; *P* (%), 17-27.68), Smith (R^2, 0.87-0.95; *P* (%), 11.74-19.84), modified Henderson (R^2, 0.86-0.91; *P*, (%) 10.96-15.82) and polynomial (R^2, 0.82-0.93; *P* (%) 18.25-29.81) models were found to inadequately fit the adsorption and desorption isotherms of whole black peppercorns (Fig. 6-3b and Table 6-3).

It can be seen in Fig. 6-4a and 6-4b that larger variability in the residuals can be observed at $a_w \geq 0.9$. There was a greater deviation between the values predicted by all the models and the experimental values. This has also been observed in modelling of the sorption isotherms of lemon peels (García-Pérez et al., 2008).

Table 6-3. Estimated parameters and fitting criteria of the models (poor fitting) applied to the experimental adsorption and desorption data of whole black peppercorns.

Models and Parameters	Adsorption			Desorption		
CAURIE	**22°C**	**30°C**	**37°C**	**22°C**	**30°C**	**37°C**
a	-0.04	-0.30	-0.22	0.19	0.51	0.49
b	4.01	4.16	3.87	3.71	3.21	3.08
R^2	0.95	0.85	0.88	0.87	0.91	0.88
R_{ave}	0.34	0.51	0.53	0.56	0.22	0.28
χ^2	6.02	14.8	9.22	18.2	6.47	7.39
P%	17.00	27.7	22.8	23.5	15.3	18.7
RSS	229	413	351	693	246	281
RMSE	2.39	3.71	2.96	4.16	2.48	2.65
SMITH						
a	-3.18	-1.88	-1.01	-0.94	-0.04	0.93
b	16.5	13.9	11.2	14.7	13.0	10.8
R^2	0.95	0.87	0.91	0.91	0.93	0.92
R_{ave}	0.00	-0.17	0.00	0.00	0.00	0.00
χ^2	5.55	11.4	6.87	11.5	4.62	4.67
P%	14.3	19.8	13.5	15.9	11.9	11.7
RSS	211.0	320	261.2	438	176	178
RMSE	2.30	3.27	2.56	3.31	2.10	2.11
Modified Henderson						
K	0.04	0.08	0.06	0.19	0.14	0.15
c	-19.6	-27.9	-33.6	-18.6	-25.8	-31.9
n	0.70	0.48	0.49	0.11	0.13	0.12
R^2	0.93	0.86	0.91	0.91	0.93	0.92
R_{ave}	-0.87	-0.68	-0.34	-0.30	-0.01	0.29
χ^2	8.29	12.8	7.37	12.1	4.75	5.06
P%	13.8	12.3	11.0	13.2	11.8	15.8
RSS	307	346	273	447	176	187
RMSE	2.77	3.40	2.61	3.34	2.10	2.16
Polynomial						
a	24.2	22.4	24.3	31.2	23.35	19.2
b	-92.5	-84.0	-86.5	-108	-78.2	-63.5
c	116	103	97.7	123	94.8	79.2
R^2	0.93	0.82	0.86	0.85	0.90	0.88
R_{ave}	0.00	-0.11	0.00	0.00	0.00	0.00
χ^2	8.38	19.1	11.0	20.7	6.91	7.72
P%	19.5	29.8	26.9	24.7	18.3	19.7
RSS	310	515	405.9	767	256	286
RMSE	2.78	4.14	3.19	4.38	2.53	2.67

The validity of a sorption model cannot be proved just by its ability to fit the experimental data. A physico-chemical (mechanistic) basis is also needed (Chirife et al., 1992). Some of the parameters of the semi-empirical GAB model have theoretical basis compared to the entirely empirical Peleg

model, which allow for the estimation of the M_0 as described previously. Despite the observation above that the Peleg model best describes the sorption isotherms of whole black peppercorns, the GAB model also performs adequately and can therefore be used to estimate the thermodynamic properties of the peppercorns. The GAB model has been reported as the best fitting model in several products i.e. potato, carrot, tomato, onion and green pepper (Kiranoudis et al., 1993), yellow dent corn (Samapundo et al., 2007a), yoghurt (Kim and Bowmilk, 1994), several fruits, vegetables and meat products (Lomauro et al., 1985) and lemon peel (García-Pérez et al., 2008). A modified GAB model was found to best describe the sorption data of maize flour (Oyelade et al., 2008). The Peleg model was reported to best describe the sorption data of rice (Toğrul and Arslan, 2006) and tea (Arslan and Toğrul, 2006).

6.3.3. Specific surface area of sorption

The values of adsorption surface area of peppercorns were calculated using the monolayer values obtained from the GAB equation. The values were 168.8, 125.6 and 123.2 m^2/g solids at 22, 30 and 37°C, respectively. The respective sorption area of the desorption were 164.9, 164.9 and 154.0 m^2/g solids. These values are within the range commonly obtained for food products (100-250 m^2/g solid) (Cassini et al., 2006). Water sorption can be influenced by surface area, composition, porosity and the number of binding sites. According to Sawhney et al. (2013), temperature has significant influence on properties of bound water and surface area of adsorbent. Increase in temperature appears to reduce the sorption area of black peppercorns in both the sorption isotherms, however, the decrease is larger during adsorption compared to desorption.

6.3.4. Isosteric heat of sorption and sorption entropy

The heat of adsorption and desorption for food materials is necessary to estimate the heat load during the drying of food materials. The a_w data generated using the GAB model was used to calculate the isosteric heat of sorption and sorption entropy. The isosteric heat of sorption and sorption entropy of both adsorption and desorption isotherms as a function of EMC are shown in Fig. 6-5.

The isosteric heat of sorption quantifies the interaction forces between the water vapour molecules and the surface of peppercorns. As can be seen in Fig. 6-5, the net isosteric heat of sorption is highly dependent on the EMC; with the energy required for sorption increasing at low moisture content and after reaching a maximum value decreasing exponentially with increasing EMC. A similar trend has been observed in previous studies (Taitano et al., 2012; Toğrul and Arslan, 2007). This could be due to the different strength of water binding. Initial occupation of the highly active polar sites on the surface could be difficult due to high interaction energy and subsequent filling of the less active sites could become possible with low energy (Arslan and Toğrul, 2005; Moreira et al., 2008). The net isosteric heat decreased rapidly until around 10% EMC and after that it decreased at very slow rate

with increasing EMC. The maximum isosteric heat for adsorption was 28.06 kJ/mol and for desorption it was 73.31 kJ/mol (Fig. 6-5) which was found at 4% EMC, which is closer to the obtained value of M_0. The isosteric heat of desorption was higher than that of adsorption at lower EMC however, the difference rapidly decreased at higher EMCs. In contrast to the crushed chillies (Arslan and Toğrul, 2005) marked difference in net isosteric heat of sorption was found between adsorption and desorption curves of black peppercorns. A similar trend has also been observed in yellow dent corn (Samapundo et al., 2007a). The information on the magnitude of heat of sorption at a particular moisture content, could give an indication on the state of the absorbed water and could help to identify the physical, chemical and microbiological stability of food.

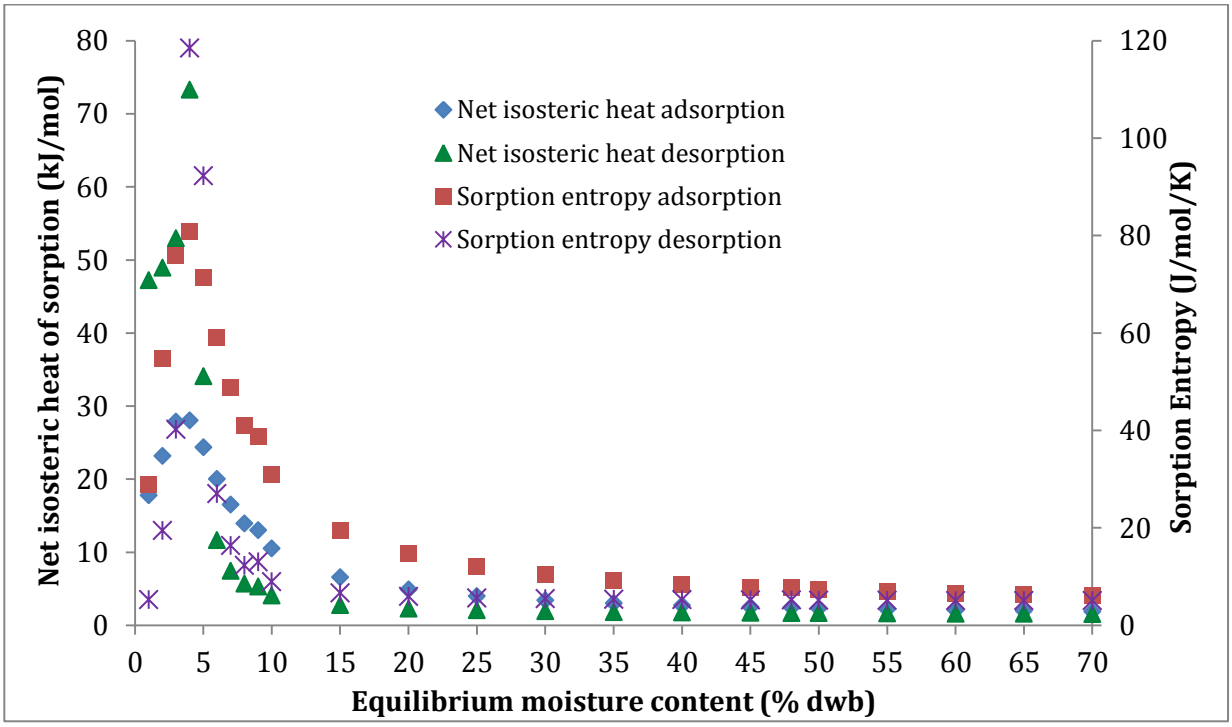

Fig. 6-5. Effect of moisture content on the net isosteric heat of sorption and sorption entropy of whole black peppercorns.

Similar to the net isosteric heat, the sorption entropy follows the same trend strongly depending on the EMC (Fig. 6-5). Yet again the entropy for desorption shows some differences to that for adsorption. Higher entropy was determined to occur during desorption than during adsorption, at 4-5% MC. The maximum sorption entropy of black peppercorns was 80.8 and 118.5 J/mol/K for adsorption and desorption, respectively. The difference rapidly decreased to become negligible at high EMCs. At around 20% EMC (dwb) both sorption curves approached to a minimum entropy and further increase in EMC resulted in adsorption and desorption having similar levels of entropy. At higher EMC the active sites on the surface could be occupied by the water molecules hence had less capacity for sorption thus reducing the entropy. Similar trends in sorption entropy has been previously reported in potatoes (McMinn and Magee, 2003), dried casein (Sawhney et al., 2011), almonds (Taitano et al., 2012), whey protein concentrate from buffalo milk (Sawhney et al., 2013)

and in sorghum (Bonner and Kenney, 2013). Moreover, the plot of enthalpy (ΔH, which includes same values as q_{st}) versus entropy (ΔS) shows a linear relationship for adsorption (R^2 0.9998) and for desorption (R^2 0.993), which indicates the existence of the enthalpy-entropy compensation. This compensation theory can be used to investigate the physical and chemical phenomena during the adsorption process (Moreira et al., 2008).

6.3.5. Gibbs free energy change and binding energy

The variability of Gibbs free energy (-ΔG) with EMC is shown for the three temperatures during adsorption in Fig. 6-6. It is clear that the (-ΔG) decreases exponentially with increasing EMC. High ΔG indicates that there is high freedom of water adsorption due to hydrophilic properties in a food material (Taitano et al., 2012). Moreover, the (-ΔG) at a specific moisture content was decreased with increase in temperature. (-ΔG) is related to the energy required in making sorption sites available, hence it becomes smaller with increase in temperature or EMC. Rate of increase in Gibbs free energy of adsorbent at higher EMCs was very slow. Above 25% EMC the curves coincided with each other, indicating minimal effect of temperatures on ΔG at higher EMC values.

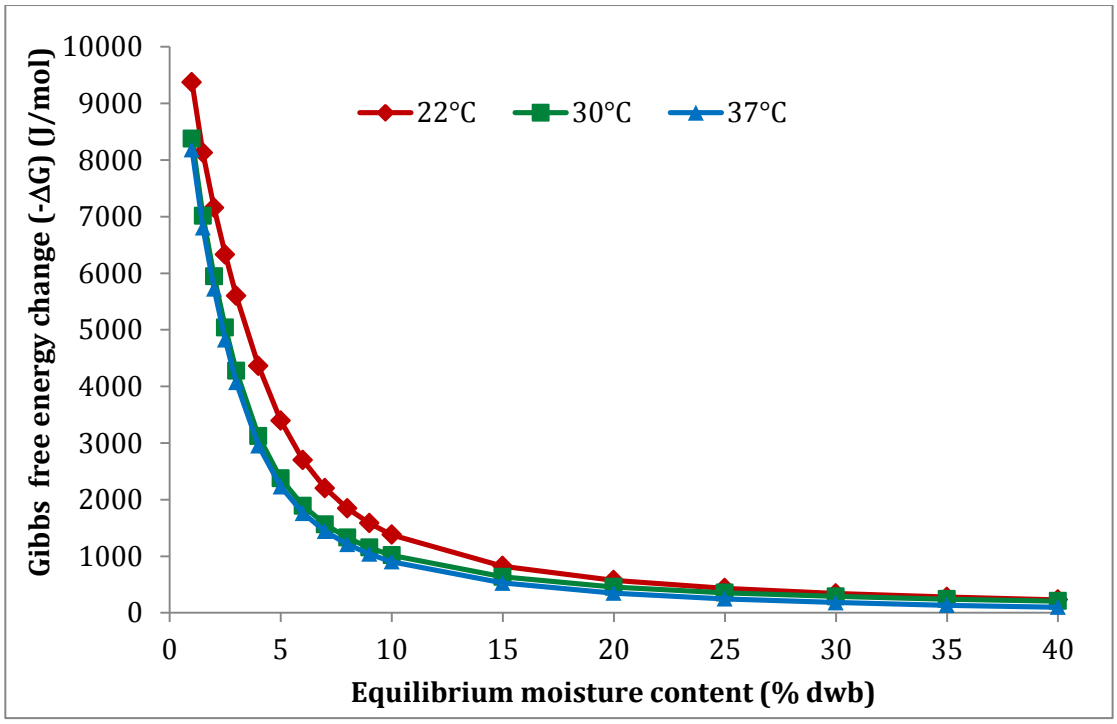

Fig. 6-6. Gibbs free energy change during adsorption of whole black peppercorns at different temperatures (22°C, 30°C and 37°C).

Similar trends were also observed with desorption, however, in contrast to adsorption a sharp decrease in (-ΔG) was observed during desorption from 4% (37,422±1880 J/mol) to 5% (6147±895 J/mol) EMC increase. Variability in ΔG during the adsorption and desorption is shown in Fig. 6-7.

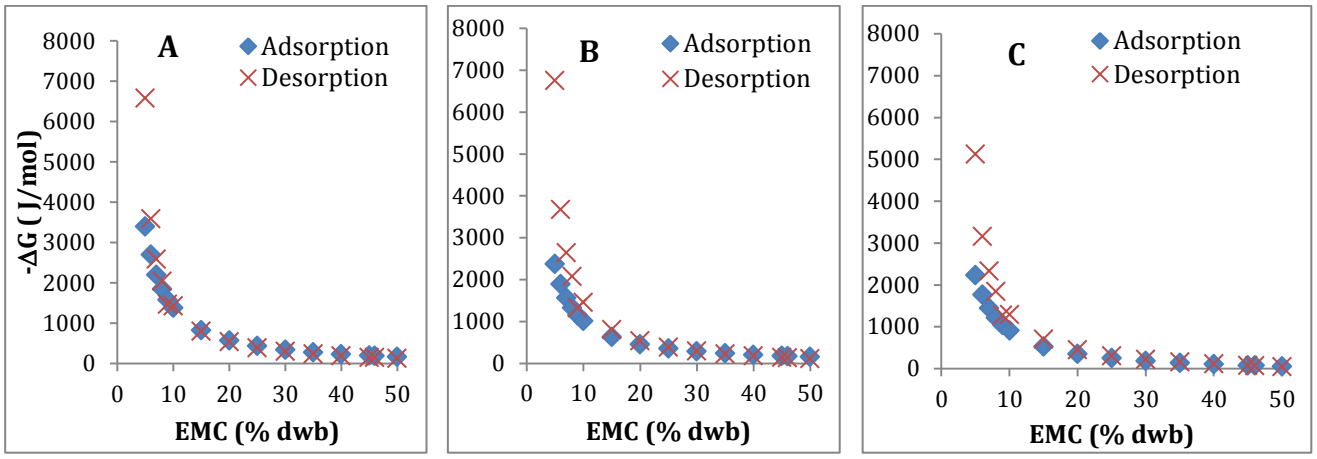

Fig. 6-7. Comparison of Gibbs free energy change during adsorption (◆) and desorption (x) of whole black peppercorns at different temperatures, A) 22°C, B) 30°C and 37°C.

Above 5% EMC the difference in free energy change was not significant between adsorption and desorption isotherm at any temperature (22°C, p=0.619; 30°C, p=0.284; 37°C, p=0.307). According to Taitano et al. (2012), the influence of MC on the (-ΔG) is more pronounced when the MC is less than the M_0. The binding energy of the primary bound water calculated using an Arrhenius plot of *ln*(C) against 1/T during adsorption was -25073.5 J/mol, which indicates that binding energy is exothermic.

6.4. CONCLUSIONS

The moisture adsorption and desorption isotherms of whole black peppercorns were successfully generated by standard static gravimetric method using different saturated salt solutions at three temperatures. The sorption isotherms were type III according to BET classification. The EMC increased with decreasing temperature at constant a_w. The EMC for desorption was generally higher than the adsorption for a particular a_w indicating the occurrence of hysteresis. Amongst the moisture sorption models evaluated, the GAB and Peleg model were found to be the best models to describe the sorption isotherms of black peppercorns over the range of temperature and a_w studied. Rather high monolayer values were obtained for desorption compared to the adsorption isotherm. The net isosteric heat of sorption and sorption entropy increased with increasing moisture content until the monolayer level and followed by an exponential drop beyond this point. These calculated thermodynamic properties could be useful in designing drying processes and storage parameters for whole black peppercorns to ensure the microbial as well as the chemical stability. Safe moisture limit of 10, 8 and 7% could be suggested for the black peppercorns at storage temperatures 22, 30 and 37°C, respectively to have a final a_w below 0.60.

The developed sorption isotherms (the best fitting GAB model) will be used for the adjustment of peppercorns to the desired a_w in **Chapter 8** for the development of predictive growth models for both *A. flavus* and *A. parasiticus* in black peppercorns.

CHAPTER

7

TOXIGENIC POTENTIALITY OF *Aspergillus flavus* AND *Aspergillus parasiticus* STRAINS ISOLATED FROM BLACK PEPPER: ASSESSED USING AN LC-MS/MS BASED MULTI-MYCOTOXIN METHOD

CHAPTER 7: TOXIGENIC POTENTIALITY OF *Aspergillus flavus* AND *Aspergillus parasiticus* STRAINS ISOLATED FROM BLACK PEPPER: ASSESSED USING AN LC-MS/MS BASED MULTI-MYCOTOXIN METHOD

Summary

A liquid chromatography triple quadrupole tandem mass spectrometry method was developed to determine mycotoxins, produced by fungal isolates grown on malt extract agar (MEA). All twenty mycotoxins produced by different fungal species (*Aspergillus, Penicillium, Fusarium and Alternaria* spp.) were extracted using acetonitrile/1% formic acid. The method was validated using the performance characteristics, linearity based on lack of fit (p=0.083-0.858), repeatability (RSDr 6.8-22.2%), intermediate precision (RSDR 10.2-24%), apparent recovery (86-113%) and limit of quantification (1.7-22.2 µg/kg). Matrix matched calibration curves were used to compensate the matrix effects and use of a single internal standard on method performance was evaluated. The developed method was applied to assess the toxigenic potentiality of *Aspergillus flavus* (n=11) and *A. parasiticus* (n=6) strains isolated from black peppers (*Piper nigrum* L.) following their growth in MEA at 22, 30 and 37°C, at colony diameter ~80-90 mm. Highest mean radial colony growth rates (μ_{max}) were observed at 30°C for *A. flavus* (5.21±0.68 mm/day) and *A. parasiticus* (4.97±0.33 mm/day). All of the *A. flavus* isolates produced aflatoxin B1 and O-methyl sterigmatocystin (OMST) while 91% produced aflatoxin B2 (AFB2) and 82% of them produced sterigmatocystin (STERIG) at 30°C. Except one, all *A. parasiticus* isolates produced all the four aflatoxins, STERIG and OMST at 30°C, but production of aflatoxin G was found to be much lower in the substrate. The following trend was found regards to mycotoxin production: 30°C>22°C>37°C, while growth rate showed a different trend, 30°C>37°C>22°C for both fungal species. Notable correlations were found between different secondary metabolites of both *Aspergillus* species.

Keywords: *Aspergillus flavus, Aspergillus parasiticus, aflatoxins, O-methyl sterigmatocystin, LC-MS/MS, malt extract agar*

Relevant publication: Yogendrarajah, P., Devlieghere, F., Njumbe Ediage, E., Jacxsens, L., De Meulenaer, B.* and De Saeger, S.* (2014). Toxigenic potentiality of *Aspergillus flavus* and *Aspergillus parasiticus* strains isolated from black pepper using an LC-MS/MS based multi-mycotoxin method. Submitted. (*Contributed equally)

7.1. INTRODUCTION

Aflatoxins are toxic secondary metabolites produced predominantly by the two species of *Aspergillus* section *Flavi*, *Aspergillus flavus* and *Aspergillus parasiticus*. Generally, *A. parasiticus* produces high concentrations of aflatoxins and most of the strains isolated (>90%) are able to synthesize aflatoxins. On the other hand, only 40-50% of the *A. flavus* isolated from natural habitats were capable of producing aflatoxins (Schmidt-Heydt et al., 2010) and according to Lisker (1993) a high incidence (77%) of aflatoxigenic strains are found among *A. flavus*. Other aflatoxin producers in *Aspergillus* section *Flavi*, include the phylogenetically closely related *A. nomius*, *A. parvisclerotigenes*, *A. bombycis*, *A. pseudotamari*, *A. minisclerotigenes*, *A. arachidicola*, and *A. toxicarius* which have been encountered less frequently (Frisvad et al., 2005; Bennet and Klich, 2003; Varga et al., 2009).

Both *A. flavus* and *A. parasticus* are usually confined in tropical and subtropical regions. According to Pitt and Hocking (1999), *A. parasiticus* was only occasionally found in South East Asia, while widely distributed in soils and foodstuffs in the United States, Latin America, South Africa, India and Australia. On the other hand, *A. flavus* is a more aggressive and widely distributed species. Thus, ecological distribution of these species could be due to the fluctuation and regional trends in climate changes, which could also reflect on the regulation of mycotoxins biosynthesis (Schmidt-Heydt et al., 2010). A very good example for this could be the recent warning for maize contamination in Europe (Northern Italy) issued in 2012-2013 as a consequence of drought conditions favourable for *A. flavus* infection (Perrone et al., 2014). Moreover, mould growth and mycotoxin contamination could be influenced by several other factors like temperature, type of substrate, water activity, inoculum concentration, microbial interaction, physiological state of the mould etc. However, the influence of these factors on growth could be different from that their influence on mycotoxin production (Garcia et al., 2009).

As previously mentioned, aflatoxin production is favored particularly by warm climates. The optimum temperatures for aflatoxin production (24-30°C) can differ among *A. flavus* and/or *A. parasiticus* isolates while optimum temperature for growth ranges from 30-35°C (Gqaleni et al., 1997; Mousa et al., 2013). Moreover, the regulation of sterigmatocystin (STERIG) and especially aflatoxins production in *Aspergillus* generally require simple sugars, low pH, reduced nitrogen source and mild oxidative stress (Georgianna and Payne, 2009). Yeast extract and sucrose enriched yeast extract (YES) agar were found to support aflatoxin production by *A. parasiticus.* Wickerhams Antibiotic Test Medium (WATM) has also been found to induce high STERIG and aflatoxins production at 25°C incubation (Georgianna and Payne, 2009). Hence, the growth medium and temperature could play a significant role in the expression of particular secondary metabolites.

Interest in the variation in aflatoxin production by *Aspergillus* section *Flavi* has increased recently because atoxigenic strains could be used as bio-control agents to reduce the aflatoxins risk (Donner et al., 2010; Abbas et al., 2011; Yu, 2012; Alaniz Zanon et al., 2013; Tran-Dinh et al., 2014; Perrone et al., 2014). Since it has been well documented that not all fungal strains are able to produce mycotoxins, this encouraged the use of modern detection and screening techniques for assessing the secondary metabolite/mycotoxin production potential of the *A. flavus* and *A. parasiticus* isolates of black pepper (*Piper nigrum* L.). Hence, a multi-mycotoxin analytical method using LC-MS/MS was developed with the prime objective to determine the secondary metabolite production by pure fungal cultures grown on malt extract agar, a growth medium widely used in mycology. The method was applied to assess the secondary metabolite production potential (AFG2, AFG1, AFB2, AFB1, STERIG and O-methyl sterigmatocystin (OMST)) of some *A. flavus* and *A. parasiticus* strains isolated from black pepper at different temperatures. Moreover, the possible correlations between different mycotoxin production were assessed. This is the first study to apply a simple and straightforward confirmatory method on secondary metabolite analysis of pure *Aspergillus* cultures grown on malt extract agar.

The developed method could be used for the assessment of mycotoxin production of other important fungi in malt extract agar. However, the main goal of this study is to select the *Aspergillus flavus* and *A. parasiticus* moulds based on their toxigenicity to be used in the development of predictive fungal growth models and to study their mycotoxin production potential at different growth conditions on peppercorns.

7.2. MATERIALS AND METHODOLOGY

7.2.1. Chemicals and reagents

Formic acid ULC-MS grade (99%) was purchased from Bio Solve BV (Dieuze, France). Phosphate-buffered saline (PBS) tablets were supplied by Oxoid (Hampshire, England). Tween 80 (polyoxyethylenesorbitan monooleate) was obtained from Merck, Germany. Other chemicals and reagents used for LC-MS and for sample preparation were of analytical grade, same as described in **Chapter 2 (section 2.2.1).**

7.2.2. Mycotoxin standards

Mycotoxin reference standards (known concentration, but not certified), O-methyl sterigmatocystin (OMST) was purchased from Chromodex (California, USA). Other mycotoxin reference standards used were the same as described before and the working standard solutions were prepared as described in **Chapter 2 (section 2.2.2).**

7.2.3. Fungal isolates, preparation of spore solution and inoculation

The strains of *A. flavus* and *A. parasiticus* used in this study were isolated from black pepper samples (n=82) collected from various markets in Sri Lanka. In total 105 strains were isolated. More details on mould isolation and characterization can be found in **Chapter 3**. Species level identification of the selected isolates (one *A. parasiticus* isolate, UG AP542 and four *A. flavus* isolates, UG AF06, AF35, AG60 and AF62) was confirmed at Mycothèque de l'Université Catholique de Louvain (MUCL, Louvain-la-Neuve, Belgium) using molecular techniques (by comparison of DNA sequence - two ad hoc genes); further information on molecular characteristics was not provided by the MUCL. Morphology of both *A. flavus* and *A. parasiticus* was generally very closely related (both belonging to *Aspergillus* section *Flavi*), however, *A. parasiticus* differ from *A. flavus* (olive green) due to the production of the very dark green colonies in *A. parasiticus*. Moreover, the presence of the rough conidial cell wall in *A. parasiticus* is the key feature to differentiate it from *A. flavus* (Samson et al., 2004). However, it was not very easy to distinguish this specific character. Hence, several identification characteristics were necessary for species level identification as mentioned in **Chapter 3**. Thus, identification of the other toxigenic *Aspergillus* isolates were confirmed by comparing with the molecularly characterised moulds using their phenotypic characteristics as well as their toxicity profile (more details are presented in section 7.3.3., Table 7-4). Eleven *A. flavus* and six *A. parasiticus* isolates were selected based on the origin, to study their toxicity profile (Table 7-5).

Spore solutions of each isolate were prepared at a concentration of 10^6 CFU/mL. Tween 80 solution (0.1 g/100 mL water), PBS (1 tablet/100 mL water) and PBS + Tween80 solution (0.1 g Tween 80 and 1 tablet PBS per 100 mL water), cotton plugs and pipette tips were autoclaved for 15 min at 121°C. To prepare the fungal inoculum, centrally inoculated MEA plates were incubated at 30°C for 10 days to enable sporulation to take place. Five mL of Tween 80 solution (wetting agent) were spread on the agar plate containing sporulated mould culture. After gently spreading the solution and scrapping off the spores, this solution was pipetted out from the agar plates and transferred to a sterile falcon tube containing a cotton plug on top for filtering out debris and mycelium. This extraction process was performed a second time on the same plate. The extracts of three agar plates were combined in one falcon tube. After removing the cotton plug, the falcon tubes were centrifuged at 8500 rpm for 15 min at 4°C. Supernatant was discarded and 20 mL of PBS + Tween 80 solution was added to the sedimented spores. After vortexing for 30 seconds, the spore solution was centrifuged again under the same conditions. After discarding the supernatant, 20 mL of the PBS solution were added to the sedimented spores and vortexed again. The spores were counted in a 16 cell thoma chamber using an inverse microscope (Olympus, IX81, Tokyo, Japan) and CellF

imaging software. Appropriate dilution was made to obtain a standardised spore concentration of ca. 10^6 spores/mL solution in PBS. This spore suspension was stored at 4°C until further use.

The basic medium used in this study was malt extract agar (MEA) (malt extract 30 g/L, mycological peptone, 5 g/L and agar 15 g/L). The medium was supplemented with chloramphenicol (100 mg/L) (Oxoid Ltd, Hampshire, England) to inhibit bacterial growth, sterilized and poured in 90 mm Petri plates (20 g/plate). The MEA plates were centrally inoculated using 10 µL of the spore solution (10^6 spores/mL) of the different *Aspergillus* isolates. The Petri plates were enclosed in a polyethylene bag (wet paper was placed inside to prevent drying of the medium) and incubated at three temperatures (22, 30 and 37°C). For each isolate, plates were prepared in triplicate.

Why only isolates from Aspergillus section Flavi were evaluated? Almost similar occurrence were found for both AFB1 and OTA in peppers (~10%) (**Chapter 3**). However, a high frequency of STERIG (40% positive of the 65 black pepper samples) contamination (11.8±3 µg/kg) was taken into account in mould selection since STERIG is a biogenic precursor of AFB1 (decision was made on the analysed 65 samples that time). The molar conversion of STERIG to AFB1 was performed using their molecular weights and total AFB1 was calculated as AFB1+STERIG equivalent to AFB1 (1 µg/kg STERIG = 0.963 µg/kg of AFB1). A worst case scenario was considered in the contamination data (ND=0; <LOD=LOD; <LOQ=LOQ) for both toxins to facilitate the selection. The concentrations of total AFB1 was thereafter compared with that of OTA. Comparing the mean concentration of the quantifiable samples, concentration of OTA was higher but with higher variability (30±31.8 µg/kg) than AFB1. The variability for AFB1 is smaller than that of OTA, and in total 20 samples were quantifiable for AFB1 compared to only 6 samples for OTA. In total, 18 of the 20 (90%) samples were above the 5 µg/kg ML for AFB1. On the other hand, 3 out of 6 (50%) were above the ML of 15 µg/kg for OTA. Comparing the median values of both mycotoxins with the EU MLs, median values of both toxins were above the ML. Moreover, the median value was above the two fold ML for AFB1 (5 µg/kg) and for OTA it was only slightly above the ML. In terms of toxicity AFB1 is a group 1 carcinogen and OTA is possibly carcinogenic (group 2B). Hence, in terms of concentration and toxicity AFB1 was found to be highly relevant compared to OTA in peppers.

Furthermore, comparing the contamination frequency, 46% of the total samples were positive for AFB1 (equivalent to AFB1+STERIG; considered positive if sample contained less or higher than LOQ of either AFB1 or STERIG) and 12% for OTA. These data analyses have shown aflatoxins were important over OTA in terms of frequency as well.

When comparing the mould contamination, the potential aflatoxin producing *A. flavus/A. parasiticus* was found in 73% of the black pepper samples while *A. niger* (62%) and *Penicillium* spp. (60%) (which could produce OTA, species level identification was not performed for *Penicllium* spp. and their toxigenicity was not studied) were found in considerable frequency. *A. ochraceus* which is generally considered as high OTA producer, was not frequently found in pepper samples as mentioned in **Chapter 3**. Few of the *A. niger* isolates (n=8) were studied for their toxigenicity. Six of these eight isolates produced FB2 and none of them produced OTA. After overall evaluation on the mycotoxins and mould contamination data, it was decided to work on the *A. flavus/A. parasiticus* and their mycotoxins in black peppers.

7.2.4. Assessment of fungal growth and lag phase

Colony growth was measured daily using an electronic digital calliper at orthogonal directions (x, y) until it reached the edge of the plate. The average of both the diameters (x, y) was recorded as the growth measurement for each isolate. Growth curves were developed by plotting the mean colony diameter (mm) of the triplicate experiments against incubation time (days). The colony growth rate (μ_{max}, mm/day) was determined from the slope of the growth curve while the lag phase (λ, days) was estimated by extrapolating the linear regression equation to the time axis. Following the growth study, the mycotoxigenic potential of all the *Aspergillus* isolates at each temperature was determined at colony diameter ~80-90 mm, when it covered the plate completely.

7.2.5. Sample preparation for mycotoxins analysis in malt extract agar

A simple and straightforward sample preparation method was developed for analysis of several fungal metabolites in MEA. Homogenised 2.0 ± 0.05 g of MEA (following the growth the whole agar content was crushed together with the fungal biomass prior to extraction) was weighed in a 50 mL extraction tube. For method validation, MEA was spiked with a mixture of mycotoxin standards at different concentrations. A fixed concentration (50 µg/kg) of zearalanone (ZAN) internal standard (IS) was added. The samples were left for an hour in the dark for equilibration. Thereafter, 10 mL of the extraction solvent (MeCN/1% formic acid (v/v)) were added and after a brief shaking, samples were extracted using an end-over-end shaker (Agitelec, J. Toulemonde and Cie, Paris, France) at position 7 for an hour. The tubes were centrifuged at 4000 g for 15 min and the supernatant was filtered using a folded filter paper into a new extraction tube. The filtrate was evaporated under N_2 at 40°C. The residue was reconstituted in 200 µL of the injection solvent (mobile phase A/B, 60/40 (v/v) mL) and centrifuged at 4000 g for 7 min. The reconstituted residue was transferred to a centrifuge filter and centrifuged at 10,000 g for 3 min. After filtration an aliquot

was transferred to the vials for LC-MS/MS analysis. Appropriate dilutions were made whenever production of mycotoxins were found to be very high following the fungal growth.

7.2.6. Instrumentation and conditions

The instrumentation (LC-MS/MS) and the conditions for mycotoxin analysis were the same as previously described in **Chapter 2 (section 2.2.5).** In addition to those mycotoxins described in **Chapter 2**, OMST was tuned, and the precursor (m/z 339), quantification (m/z 306) and qualification (m/z 324) ions were monitored (Table 7-1; Fig. 7-1).

Table 7-1. Parameters for the mass spectrometric detection of mycotoxins, analyte retention time (tR), precursor ions, pseudo-molecular ion, cone voltage (CV), quantification (Quan), qualification (Qual) ions and collision energy (CE).

Mycotoxins	tR (min)	Precursor ion (m/z)	Pseudo-molecular ion	CV (V)	Quan (m/z)	Qual (m/z)	CE (eV)
OMST	6.04	339.0	$[M+H]^+$	52	306.0	324.0	28 25
ZEN	7.40	319.1	$[M+H]^+$	27	187.2	283.3	15 20
ZAN (IS)	7.51	321.0	$[M+H]^+$	27	303.3	189.2	13 19

OMST, O-methyl sterigmatocystin; ZEN, Zearalenone; ZAN (IS), Zearalanone (Internal Standard)

7.2.7. Matrix effect evaluation

The matrix effect (ME) was evaluated by comparing the peak responses of the standard mycotoxins (n=3) spiked in the extraction solvent with the spiked MEA extracts at five concentration levels for each analyte. A standard mixture of mycotoxins was prepared using the individual stock and working standard solutions at the following concentrations for determining the ME: AFB1, AFB2, AFG1, AFG2 and OMST (0.5 µg/mL), OTA and ROQ C (1.0 µg/mL), STERIG (0.625 µg/mL), T-2, HT-2, NEO, 3-ADON, 15-ADON, AME and CIT (2.5 µg/mL) and DON, FB1, FB2, FB3 (5 µg/mL). The ME was calculated using the formula described in **Chapter 2 (section 2.2.6).**

7.2.8. Method validation

The multi-mycotoxin analytical method for MEA was validated using spiked blank MEA samples. A set of performance characteristics that were in compliance with the recommendations and guidelines defined by the Commission Decision 2002/657/EC (EC, 2002) and Regulation EC/401/2006 (EC, 2006a) were evaluated. Validation parameters assessed were, linearity, recovery, limit of detection (LOD), limit of quantification (LOQ), intra-day repeatability (RSDr) and inter-day reproducibility (intermediate precision; RSDR). Calculations based on both peak area (absolute response (AR)) and relative peak area (relative response (RR)) were used to evaluate or compare

the performance criteria of the method developed. Relative response was calculated by dividing the absolute peak area of the analyte by the peak area of internal standard ZAN.

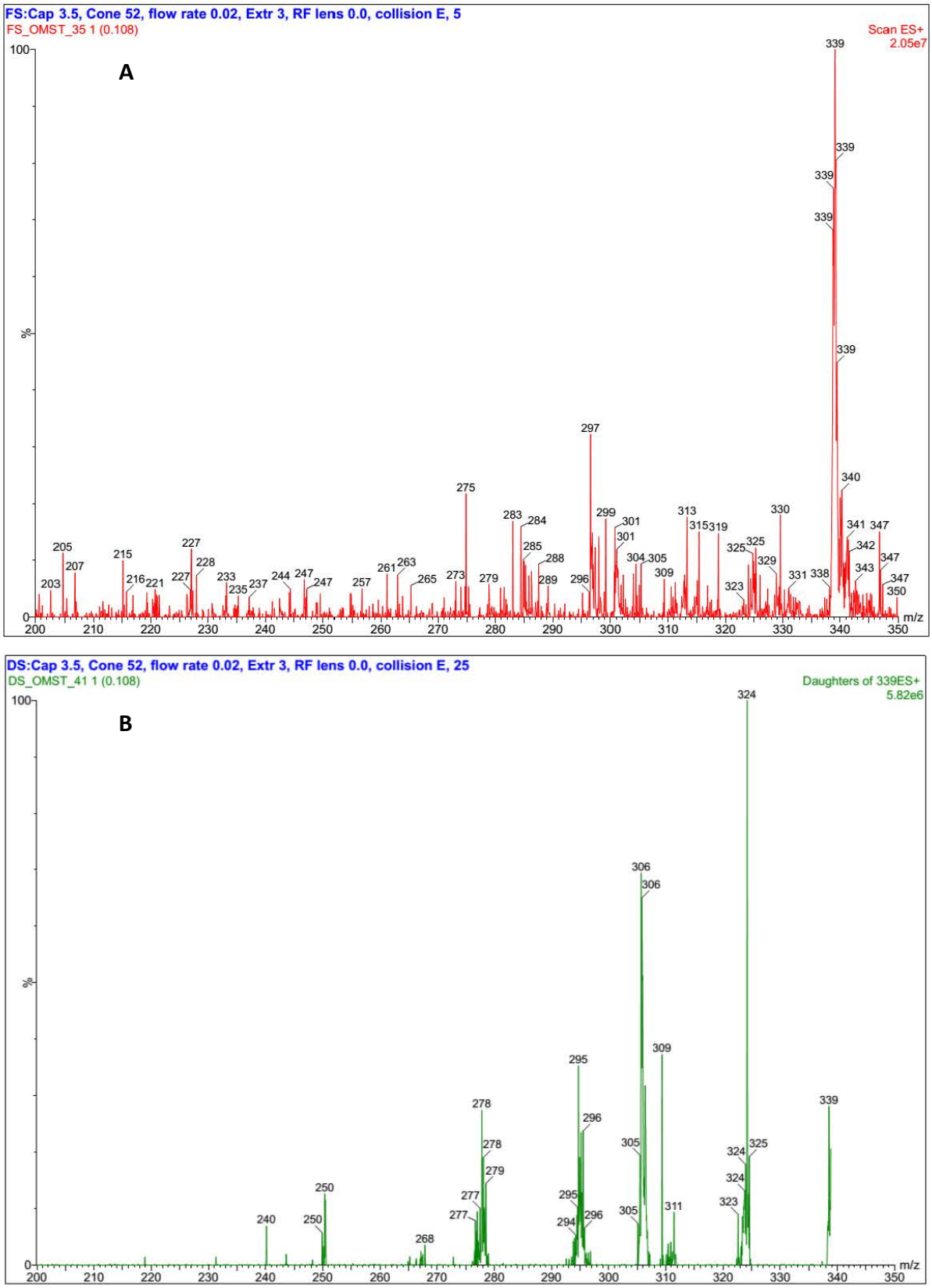

Fig. 7-1. A. Full scan of O-methyl sterigmatocystin (OMST) at cone voltage 52V showing the abundant precursor ion m/z 339 and B. Daughter scan of m/z 339 fragmentation at cone voltage 52V and collision energy 25eV showing the most abundant product ion m/z 324 and the second most abundant product ion m/z 306.

7.2.8.1. Calibration curves, linearity, LOD, LOQ and recovery

Linearity was evaluated using matrix matched calibration (MMC) curves, by spiking blank MEA at six concentration levels. Calibration curves were constructed by plotting the analyte response (absolute or relative response (y)) versus the concentration of analyte (x).

The concentration ranges used for this validation study were*:* AFs and OMST (2.5-20 µg/kg); OTA and ROQ C (5-40 µg/kg); T-2, HT-2, NEO, 3-ADON, 15-ADON, ZEN and CIT (12.5-100 µg/kg), STERIG (3.125-25 µg/kg), AME, DON, FB1, FB2 and FB3 (25-200 µg/kg). Calculations were performed separately on absolute and relative peak responses (n=6). Linear regression was used to fit the calibration curve and lack of fit test was used to assess the fitting of the regression model. LOD and LOQ were determined using the MMC curves prepared in MEA using the same procedure as described in **Chapter 2 (section 2.2.7.1)**. Recovery of the method was calculated using the validation experiments that were used to calculate the LODs and LOQs. Apparent recovery was determined by calculating the ratio of the predicted value obtained from the MMC curves to the actual/theoretical value (Sulyok et al., 2006).

7.2.8.2. Intra-day repeatability and intermediate precision

Intra-day repeatability was calculated via relative standard deviations (RSD) to explain the same day variability. The intermediate precision/inter-day reproducibility which could explain the total variability of the method was calculated using the analysis of variance (ANOVA) approach.

7.2.9. Statistical analysis

One way ANOVA, lack of fit test, non-parametric Mann-Whitney U test and Kruskal Wallis one way ANOVA were performed using the SPSS statistical software (IBM®, Version 22). Level of significance (α) was 0.05, unless otherwise specified.

7.3. RESULTS AND DISCUSSION

7.3.1. Method development and matrix effect evaluation

A straight forward extraction procedure using acidified acetonitrile (1% formic acid) was performed to extract multi-class secondary metabolites that could be produced by different fungal species (*Aspergillus, Penicillium, Fusarium* and *Alternaria* spp.) from MEA. All twenty analytes were simultaneously detected in a run time of 20 min using ESI (+)ive mode. All the metabolites however eluted before 10 min, leaving sufficient time for column cleaning and regeneration. Having close retention times or co-elution was not a problem, since analyte specific ions were fixed for quantification and confirmation using MRM mode, thus selectivity was not compromised. Previous studies using rather complex extraction procedures for YES agar, failed to extract

STERIG, one of the major metabolites of the aflatoxin biosynthetic pathway and reported very high LOQs for a number of metabolites (Van Pamel et al., 2011).

Matrix effects are common problems that occur when using LC-MS or LC-MS/MS, and thus have an adverse effect on the analytical results. The response of the target compound can be enhanced or suppressed due to the interfering matrix components, which is commonly known as signal suppression/enhancement effect (SSE). The ME due to the co-extractives from MEA on different metabolites is shown in Fig. 7-2. A comparison was made between the absolute response (AR) and relative response (RR) on matrix effect. The ME for most of the analytes ranged from (-15.6) to 34.7% and from (-28.3) to 1.71% based on AR and RR calculations, respectively. Strong signal suppression was observed with DON in both the approaches (AR: -42.2% and RR: -51.2%). A range in between (-20) to +20% ME or SSE in between 0.8 to 1.2 was generally considered as tolerable (Frenich et al., 2011). Based on the AR, 90% of the analytes were within this acceptable ME range, while considering their RR 75% of the analytes were inside this range. Values outside this range indicate severe ME as it has been observed with DON and CIT when using AR and with DON, 3-ADON, AFG2, AFG1, AFB2 and AFB1 when using RR. This could explain the insignificant contribution of the IS used in compensating the ME of these analytes. However, significantly low ME was obtained for some other metabolites like T-2, AME and CIT when RR was used for ME evaluation (Fig. 7-2).

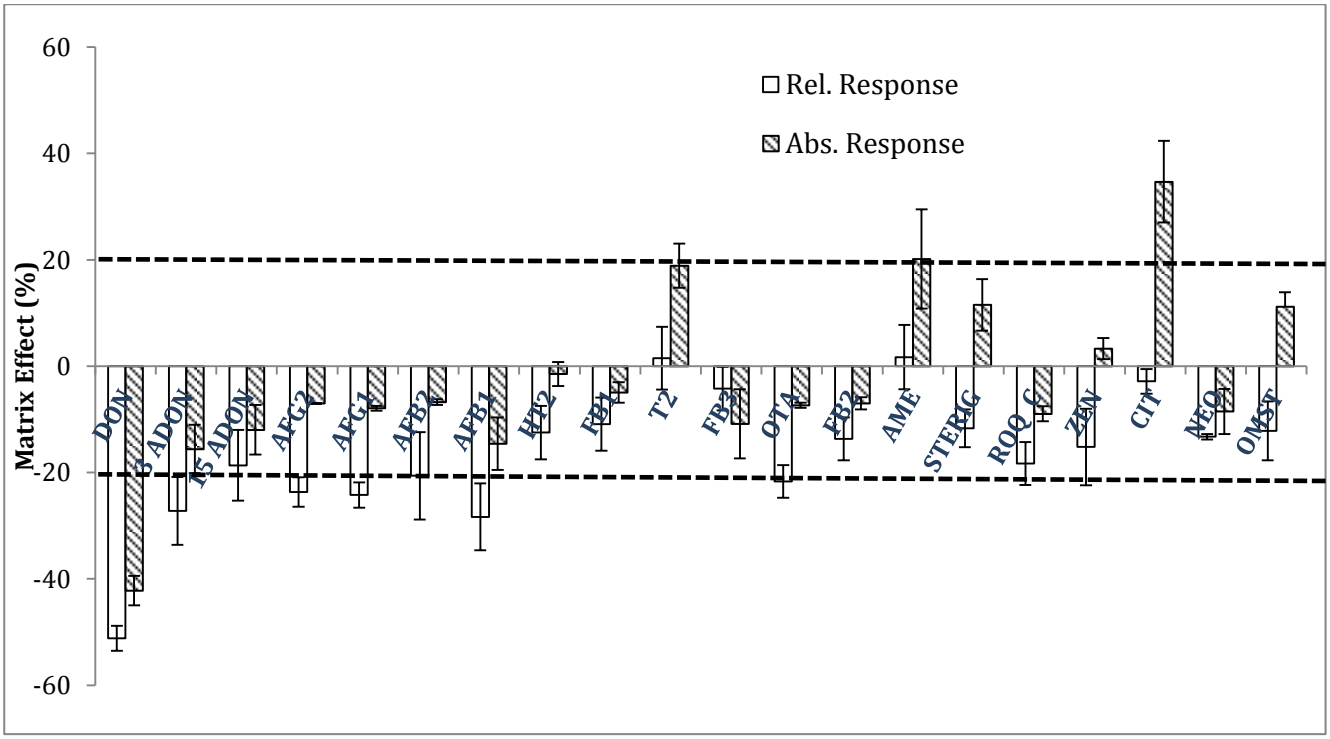

Fig. 7-2. Matrix effect of different mycotoxins in malt extract agar; a comparison based on absolute versus relative analyte response. A tolerance level of matrix effect is shown between the two dashed lines.

Though ZAN is the most appropriate IS for ZEN (in terms of its similar chemical property and elution closer to the retention time of ZAN), the ME for ZEN is still rather high compared to calculations using AR. The best option to tackle matrix effects is the use of isotopically labeled IS, lacking those, structural analogues could be the second best option. However, this adds cost and finding structural analogues of each metabolite has never been easy. Hence, in this study MMC curves were used to compensate these variable ME and to improve the linearity, reliability and accuracy of the analytical results of the developed method.

7.3.2. Performance characteristics of the method

Method validation was performed in terms of linearity (lack of fit), LODs, LOQs, recovery, repeatability and intermediate precision. The performance characteristics were compared using both absolute and relative responses.

7.3.2.1. Linearity, LOD, LOQ and recovery

MMC curves developed on blank MEA matrices were linear over the working concentration ranges in all of the studied mycotoxins. Calibration curves were fitted by linear regression and the linearity was assessed using the p-values of the lack of fit test (Table 7-2). Lack of fit p-values were in the range of 0.083-0.858 and 0.063-0.755 based on the absolute and relative response calculations, respectively. P-values greater than 0.05 are considered as there is no lack of fit, thus showing good fit of the model for all the studied analytes. There were no significant differences ($p=0.562$) in the mean lack of fit values of different analytes, in both calculations at 5% level of significance. This explains that that there is no significant contribution of the IS used in "good" fitting of the linear regression of different analytes thus, either absolute or relative response could be used in fitting the calibration curves in this method. Additionally, residual plots of each mycotoxin were assessed to ensure decent fit of the data to the linear model.

Moreover, mean apparent recoveries for all the tested mycotoxins were in the range of 86-113% (Table 7-2), within the acceptable range of required performance criteria (EC, 2006a). There were no significant differences in apparent recoveries ($p=0.561$) between different analytes based on both absolute or relative response estimations. Associated variability is minimum probably because the sample preparation procedure applied is rather simple avoiding long clean-up steps; thus losses could be marginal.

Table 7-2. Performance criteria of the developed multi-analyte method; a comparison based on the calculations of absolute versus relative response.

Mycotoxin	Absolute Response		Relative Response	
	Lack of fit (p-value)	Apparent recovery (%)	Lack of fit (p-value)	Apparent recovery (%)
DON	0.125	95	0.215	92
3-ADON	0.706	111	0.539	112
15-ADON	0.244	113	0.722	111
AFG2	0.078	104	0.166	110
AFG1	0.289	95	0.600	98
AFB2	0.29	106	0.212	107
AFB1	0.397	97	0.747	94
HT-2	0.282	109	0.755	106
FB1	0.338	100	0.461	104
T-2	0.536	102	0.201	113
FB3	0.858	109	0.406	110
OTA	0.241	108	0.168	111
FB2	0.083	110	0.187	108
AME	0.418	108	0.699	110
STERIG	0.441	93	0.331	101
ROQ C	0.711	113	0.627	100
ZEN	0.662	105	0.520	105
CIT	0.179	86	0.210	87
NEO	0.186	97	0.063	111
OMST	0.506	103	0.503	96

The LODs and LOQs of different analytes ranged from 0.8-14.6 and 1.7-29.2 µg/kg, respectively (Table 7-3) based on AR. The purpose of this analytical method is to quantify the production of several secondary metabolites following the growth of pure fungal cultures (generally they produce in high concentrations in agar under optimal conditions). Most of the LOQs obtained with this method were quite low hence, this method could be useful in studying the wide range of metabolite production of several fungi (*Aspergillus*, *Fusarium*, *Penicllium* and *Alternaria* spp.) and also to identify the non-toxigenic ones for potential use in mycotoxins bio-control. Since many different metabolites were extracted with this single solvent, this extraction procedure as it is or with slight modification (with some water) can be used also in untargeted analysis.

7.3.2.2. Intra-day repeatability and intermediate precision

Relative standard deviations (RSD) were calculated at intra-day repeatability (RSDr) and inter-day reproducibility (RSDR) conditions. The results are summarized in Table 7-3. RSDr values were

within the acceptable range of <20%, matching with the performance criteria requirement of the EC (2006a) except for few analytes (DON and ADONs (19.4-22.2%)). RSDr ranged from 6.8% to 22.2% and 8.6-23.5% based on absolute and relative response, respectively. The results were much comparable between both approaches, except CIT and NEO which had much lower RSDr (CIT 6.8% and 20.7%; NEO 10.9% and 20.3%) values based on the absolute response compared to the relative ones.

Table 7-3. Limit of detection (LOD) (µg/kg), limit of quantification (LOQ) (µg/kg), repeatability (RSDr) (%) and intermediate precision (RSDR) (%) of different mycotoxins in malt extract agar determined based on the absolute and relative response.

Mycotoxin	Absolute response				Relative response			
	LOD	LOQ	RSDr	RSDR	LOD	LOQ	RSDr	RSDR
DON	6.4	12.9	20.3	24.0	10.1	20.2	23.5	25.8
3-ADON	3.5	7.0	19.4	22.6	6.8	13.6	18.9	22.8
15-ADON	2.4	4.7	22.2	23.4	7.3	14.6	18.5	19.1
AFG2	1.4	2.9	17.5	17.7	1.4	2.8	13.4	13.7
AFG1	1.8	3.6	14.1	16.8	1.1	2.2	22.1	23.3
AFB2	0.8	1.7	15.4	21.3	0.9	1.8	14.9	28.5
AFB1	1.3	2.6	9.4	14.5	1.3	2.6	13.5	15.3
HT-2	3.3	6.7	16.9	17.8	3.7	7.4	15.8	17.0
FB1	5.7	11.5	17.6	19.7	3.7	7.3	15.1	22.4
T-2	1.6	3.2	14.2	16.8	2.6	5.3	9.8	13.1
FB3	7.4	14.7	19.8	19.7	8.8	17.7	12.6	23.1
OTA	1.8	3.6	19.0	15.9	3.7	7.4	8.6	13.0
FB2	7.1	14.2	17.5	20.3	8.1	16.2	12.1	22.1
AME	5.7	11.5	14.4	14.6	2.6	5.3	9.2	15.1
STERIG	0.9	1.8	19.6	21.1	0.9	1.7	9.4	10.2
ROQ C	1.9	3.9	13.5	18.3	1.1	2.2	19.1	23.7
ZEN	3.3	6.6	14.8	15.4	3.1	6.2	13.1	16.0
CIT	14.6	29.2	6.8	19.7	5.9	11.8	20.7	21.4
NEO	6.2	12.5	10.9	23.4	2.4	4.9	20.3	24.9
OMST	1.3	2.6	13.0	18.2	1.6	3.2	12.6	16.4

Considering the intermediate precision/reproducibility, the RSDR values based on absolute and relative response ranged from 14.5-24% and 10.2-28.5%, respectively. There were no significant differences in RSDr ($p=0.195$) or RSDR ($p=0.951$) when comparing the values calculated either using AR or RR. Hence, statistical comparison of all the method performance parameters shows that the quantification of most of the analytes can be performed either using absolute or relative responses.

To assess the applicability of the developed method different *Aspergillus* isolates were used in this study. Moreover, aflatoxins have shown smaller signal suppression when using absolute response compared to the relative thus absolute response of the MMC curves was used for quantification of the metabolites produced by these fungi.

7.3.3. Growth assessment of the fungal isolates at different temperatures

Of the 105 isolates of *A. flavus* and/or *A. parasiticus* from black pepper, 38 (36%) of them were found to be capable of producing toxins to different extents and the remaining 67 (64%) were atoxigenic. It has to be stated that hey have been grouped as atoxigenic based on their inability to produce aflatoxins, OMST and STERIG. Cyclopiazonic acid (CPA) production was not evaluated. From the 38 toxigenic isolates, 29 of them were identified as *A. flavus* while 9 were *A. parasiticus.* More details on the toxicity profile of these fungal isolates are presented inTable 7-4.

Table 7-4. Toxigenic profile and origin of *Aspergillus* section *Flavi* strains isolated from black pepper samples from Sri Lanka (mycotoxins were analyzed after 14 days of incubation at 30°C in malt extract agar).

No	Strain ID	AFG2	AFG1	AFB2	AFB1	STERIG	OMST	Chemotype	Origin
1	UG AF11	x	x	✓	✓	✓	✓	AFB producer	Kandy/Matale
2	UG AF54	x	x	✓	✓	x	✓	AFB producer	Anuradhapura
3	UG AF60*	x	x	✓	✓	✓	✓	AFB producer	Kandy/Matale
4	UG AF62*	x	x	✓	✓	✓	✓	AFB producer	Kandy/Matale
5	UG AF64	x	x	x	✓	x	✓	AFB producer	Kandy/Matale
6	UG AF93	x	x	✓	✓	✓	✓	AFB producer	Anuradhapura
7	UG AF37	x	x	✓	✓	x	✓	AFB producer	Jaffna
8	UG AF36	x	x	✓	✓	✓	✓	AFB producer	Jaffna
9	UG AF201	x	x	x	✓	x	nc	AFB producer	Kandy/Matale
10	UG AF112	x	x	x	✓	✓	✓	AFB producer	Kandy/Matale
11	UG AF06*	x	x	✓	✓	✓	✓	AFB producer	Kandy/Matale
12	UG AF56	x	x	x	✓	x	nc	AFB producer	Anuradhapura
13	UG AF351	x	x	✓	✓	x	nc	AFB producer	Jaffna
14	UG AF35*	x	x	✓	✓	✓	✓	AFB producer	Jaffna
15	UG AF792	x	x	x	✓	✓	x	AFB producer	Anuradhapura
16	UG AF411	x	x	x	✓	x	✓	AFB producer	Jaffna
17	UG AF202	x	x	x	✓	✓	nc	AFB producer	Kandy/Matale
18	UG AF111	x	x	x	✓	✓	✓	AFB producer	Kandy/Matale
19	UG AF362	x	x	✓	✓	✓	✓	AFB producer	Jaffna
20	UG AF13	x	x	✓	✓	✓	nc	AFB producer	Kandy/Matale
21	UG AF352	x	x	✓	✓	✓	✓	AFB producer	Jaffna
22	UG AF23	x	x	✓	✓	✓	nc	AFB producer	Jaffna
23	UG AF241	x	x	✓	✓	✓	nc	AFB producer	Jaffna
24	UG AF29	x	x	x	✓	✓	nc	AFB producer	Jaffna
25	UG AF861	x	x	✓	✓	✓	✓	AFB producer	Kandy/Matale
26	UG AF79	x	x	✓	✓	✓	nc	AFB producer	Anuradhapura
27	UG AF85	x	x	x	✓	✓	nc	AFB producer	Anuradhapura
28	UG AF242	x	x	x	✓	✓	nc	AFB producer	Jaffna
29	UG AF82	x	x	✓	✓	x	✓	AFB producer	Kandy/Matale

30	UG AP461	✓	x	✓	✓	✓	✓	AFB+G producer	Kandy/Matale
31	UG AP53	✓	x	✓	✓	✓	nc	AFB+G producer	Kandy/Matale
32	UG AP631	✓	x	✓	✓	✓	✓	AFB+G producer	Kandy/Matale
33	UG AP821	✓	x	✓	✓	x	✓	AFB+G producer	Kandy/Matale
34	UG AP51	✓	x	✓	✓	✓	✓	AFB+G producer	Kandy/Matale
35	UG AP28	✓	x	x	✓	✓	✓	AFB+G producer	Jaffna
36	UG AP542*	✓	✓	✓	✓	✓	✓	AFB+G producer	Anuradhapura
37	UG AP462	✓	x	✓	✓	✓	✓	AFB+G producer	Anuradhapura
38	UG AP61	✓	x	✓	✓	✓	✓	AFB+G producer	Kandy/Matale

*Species level identification of these isolates were confirmed at MUCL based on the molecular techniques, only these isolates were used in predictive mycology in Chapter 8 and for inhibition study in Chapter 9. Identification of the other isolates were confirmed based on the phenotypic characterization (using identification keys), and toxicity profile after comparing it with those of the molecularly characterized isolates; nc-production was not confirmed at this screening stage.

This study focused further on the growth and mycotoxin production potential of eleven *A. flavus* (Kandy/Matale-5; Anurdhapura-2; Jaffna-4) and six *A. parasiticus* (Kandy/Matale-3; Anurdhapura-2; Jaffna-1) isolates (Table 7-5). Selection was done on the basis of the origin of the sample.

Table 7-5. Maximum radial growth rates (μ_{max}) (mean±SD, mm/day) and lag phases (λ) (mean±SD, days) of *A. flavus* (A) and *A. parasiticus* (B) isolates of black pepper grown on malt extract agar and incubated at 22, 30 and 37°C. The origin of each *Aspergillus* isolate (sampling district in Sri Lanka) is also given.

Strain ID	Origin of the *Aspergillus* isolate	Temperature (°C)					
		22		30		37	
		μ_{max}	λ	μ_{max}	λ	μ_{max}	λ
		A. Aspergillus flavus					
UG AF861	Kandy/Matale	2.67±0.48	1.23±0.34	5.20±0.14	0.87±0.02	3.68±0.81	3.13±0.06
UG AF60	Kandy/Matale	2.75±0.45	1.32±0.37	5.00±0.04	0.85±0.08	3.43±0.47	2.65±0.95
UG AF93	Anuradhapura	2.35±0.09	0.92±0.23	4.97±0.54	0.92±0.03	2.95±0.84	2.54±0.92
UG AF36	Jaffna	2.71±0.20	1.32±0.11	5.77±0.36	0.89±0.03	3.57±0.92	2.94±0.25
UG AF62	Kandy/Matale	2.74±0.17	1.16±0.04	5.30±0.64	0.80±0.14	4.79±0.28	3.13±0.03
UG AF54	Anuradhapura	2.70±0.13	1.24±0.14	5.24±0.57	0.81±0.16	4.56±0.19	3.12±0.02
UG AF411	Jaffna	3.08±2.14	0.73±0.33	5.53±0.94	0.83±0.06	4.26±2.37	2.90±0.46
UG AF82	Kandy/Matale	4.50±2.07	0.91±0.42	4.07±1.30	0.74±0.17	2.57±1.77	2.90±0.44
UG AF06	Kandy/Matale	2.87±0.36	1.18±0.22	5.79±0.16	0.90±0.05	3.95±0.32	3.14±0.03
UG AF362	Jaffna	2.44±0.06	0.86±0.22	5.69±0.17	0.92±0.09	4.67±0.56	3.11±0.06
UG AF35	Jaffna	2.69±0.17	1.21±0.05	5.49±0.38	0.86±0.07	4.38±0.64	3.08±0.04
		B. Aspergillus parasiticus					
UG AP821	Kandy/Matale	2.52±0.07	1.25±0.15	5.15±0.37	0.90±0.04	2.54±0.56	3.30±0.37
UG AP631	Kandy/Matale	2.42±0.43	1.20±0.19	5.67±0.40	0.87±0.04	4.33±0.06	3.08±0.05
UG AP61	Kandy/Matale	2.16±0.40	0.61±0.64	5.67±0.23	0.90±0.02	4.20±1.77	2.97±0.22
UG AP542	Anuradhapura	2.10±0.38	0.54±0.63	5.21±0.16	0.85±0.05	2.15±1.75	3.68±1.52
UG AP462	Anuradhapura	2.47±0.11	1.19±0.05	5.22±0.04	0.85±0.02	3.88±0.50	3.07±0.05
UG AP28	Jaffna	2.57±0.19	1.08±0.16	5.90±0.28	0.86±0.09	4.93±0.38	3.00±0.12

Different isolates of the same species (*A. flavus* or *A. parasiticus*) showed almost similar growth rates at a particular temperature (Table 7-4). A slightly higher growth rate was found only with one *A. flavus* isolate UG AF82 (4.50±2.07mm/day) at 22°C. Moreover, both *A. flavus* and *A. parasiticus* isolates showed a similar growth responses at a particular temperature (Table 7-4). For both species the highest mean radial colony growth rates (μ_{max}) were observed at 30°C (mean±SD 5.21±0.68 mm/day for *A. flavus* and 4.97±0.33 mm/day for *A. parasiticus*) for most of the isolates, followed by those at 37°C (4.00±0.86 mm/day for *A. flavus* and 4.50±1.00 mm/day for *A. parasiticus*). Generally, the lowest mean growth rate s of all the isolates observed at 22°C (2.79±0.70 mm/day for *A. flavus* and 2.48±0.33 mm/day for *A. parasiticus*). Depending on the type of strain, at 30°C the colonies reached the diameter of ~80-90 mm in ca. 7-9 days. At 37°C it took ca. 11-12 days while at 22°C it took ca. 14-16 days. Generally, inter/intra-species differences in growth rate were small at a particular temperature. The distributions of the medians of growth rates and lag phases between the different temperature groups were significantly different ($p<0.001$) in both species. The findings are consistent with other studies showing that the optimum temperature for the growth of *A. parasiticus* on MEA is 31°C (Garcia et al., 2011), while for *A. flavus* on other types of synthetic culture media it is 32-36°C depending on the substrate and the isolate (Astoreca et al., 2012).

7.3.4. Toxigenic potential of the fungal isolates at different temperatures

The number of fungal isolates of each species producing different mycotoxins at different temperatures (22, 30 and 37°C) are shown in Table 7-6. Capability of toxic secondary metabolites production (toxigenic potential/"toxigenicity") by various isolates of *A. flavus* (AFB2, AFB1, OMST and STERIG) and *A. parasiticus* (AFG2, AFG1, AFB2, AFB1, OMST and STERIG) isolates incubated at different temperatures is shown in Tables 7-7 and 7-8, respectively. MRM chromatograms showing the production of all the four aflatoxins, STERIG and OMST by an *A. parasiticus* isolate and the production of AFB2, AFB1, OMST and STERIG by an *A. flavus* isolate are shown in Fig. 7-3A and 7-3B, respectively.

7.3.4.1. Toxigenic potential of *A. flavus* isolates

All *A. flavus* isolates produced AFB1 and OMST while 91% produced AFB2 and 82% produced STERIG (it is possible that the STERIG produced by the other two isolates has been already biotransformed to OMST and finally to AFB1) at 30°C (Table 7-6). STERIG, the carcinogenic polyketide is the penultimate intermediate in the aflatoxin biosynthetic pathway. It is converted to OMST prior to the production of AFB1 or AFG1 (Rank et al., 2011; Cleveland et al., 2009). For additional understanding a detailed aflatoxins biosynthetic pathway is provided in **Chapter 1** (Fig. 1-5).

A large variability in toxigenicity was observed among different isolates of the same species grown on MEA at a particular temperature (Table 7-7). Marín et al. (2008b), emphasized that high variability exists in mycotoxin production by a given strain in a given substrate. High variability in mycotoxin production by *Alternaria* spp. was also observed in tomato (Van de Perre, 2014). In our study, the production of mycotoxins by the *Aspergillus* isolates was found to be highly temperature dependent; a large number of isolates both *A. flavus* and *A. parasiticus* produced high concentrations of mycotoxins at 30°C. The number of isolates producing mycotoxins and the concentration of the mycotoxins produced were very small at 37°C compared to the other two temperatures. At 37°C only four isolates of *A. flavus* produced AFB1, but at very low concentrations (0-42.1 µg/kg) compared to the production at 30°C (up to 8004.5±1563.4 µg/kg). These findings are in agreement with other studies in which both *A. flavus* and *A. parasiticus* had optimum temperature for growth and mycotoxin production around 30°C, however, different growth media were used in those studies (Mousa et al., 2013; Garcia et al., 2011; Schmidt-Heydt et al., 2010; Lozano-Ojalvo et al., 2013). According to Bhatnagar et al. (2006), biosynthesis of aflatoxins by *A. flavus* is optimal at temperatures between 29 and 30°C, and is significantly decreased at temperatures <25°C and >37°C (O'Brian et al., 2007).

Table 7-6. Number (percentage) of fungal isolates of each *Aspergillus* species producing mycotoxins at temperatures 22, 30 and 37°C (mycotoxin production was determined at colony diameter ~80-90 mm, when plate was completely covered by the colony).

Fungal species	Temperature (°C)	AFG2	AFG1	AFB2	AFB1	STERIG	OMST
A. flavus (n=11)	22	0	0	7 (64)	8 (73)	7 (64)	9 (82)
	30	0	0	10 (91)	11 (100)	9 (82)	11 (100)
	37	0	0	1 (9)	4 (36)	3 (27)	3 (27)
A. parasiticus (n=6)	22	1 (17)	1 (17)	5 (83)	5 (83)	5 (83)	5 (83)
	30	6 (100)	5 (83)	5 (83)	6 (100)	5 (83)	6 (100)
	37	2 (33)	1 (17)	1 (17)	1 (17)	1 (17)	1 (17)

Among the eleven *A. flavus* isolates studied, UG AF93, AF60, AF54, AF06 and AF35 were identified as high mycotoxin producers. Some *A. flavus* isolates (UG AF60, AF06 and AF35) produced extremely higher concentrations of AFB1 at 22°C (max. 16-40 mg/kg) than at 30°C (max. 10 mg/kg) and the least production was observed at 37°C (max. 42 µg/kg). The isolates AF93, AF36 and AF54 also produced rather high AFB1 at 22°C. High mycotoxin production at 22°C could be explained by the fact that under high a_w conditions (agar) the optimum temperature for

aflatoxin production can vary a lot, depending on the strain (Klich et al., 2007). Also the type of growth medium could play a role. In agreement with O'Brian et al. (2007), all the *A. flavus* isolates were producing significantly low concentration of metabolites (or no production) at 37°C. In our study, 64% of the *A. flavus* isolates did not produce any of the studied secondary metabolites at 37°C. Difference in intensity of sporulation (not quantified) was also observed between different temperatures though sporulation initiated at different periods. Previous studies have shown that temperature affects aflatoxin production and the transcriptional profile of *Aspergillus* (O'Brian et al., 2007). At elevated temperatures of 37°C, one or more pathway enzymes become non-functional due to their significant reduction in transcription, leading to failure of the strains to produce the toxins. Therefore, it can be concluded that mycotoxin production is very much temperature dependent, as well as strain specific.

Despite a similar growth response and sporulation with other *A. flavus* isolates, the isolate UG AF861 was found to be the weakest producer of mycotoxin in this medium followed by UG AF411. The isolates UG AF861 and UG AF411 did not produce STERIG at any temperature but both produced small quantities of OMST and AFB1 at levels lower than the limit of quantification of the method (1.3 µg/kg). Probably, the little amount of STERIG produced had already been bio-transformed to OMST.

Generally, the growth rates of these isolates at 37°C were lower than at 30°C but higher than at 22°C. It was not possible to find an association between secondary metabolite production and growth rate of a particular isolate. In previous studies poor correlation has been observed between growth and mycotoxin production and the relationship between the rates of primary and secondary metabolism is still not clear (Garcia et al., 2009). Moreover, production of mycotoxins by a particular fungal isolate also varied between replicates even at the same growth conditions. This makes the prevention, control and regulation of fungal secondary metabolism a very challenging topic to understand till today.

Table 7-7. Mycotoxin production (mean±SD µg/kg) of *A. flavus* strains isolated from black pepper following the growth in malt extract agar at different temperatures (determined at colony diameter ~80-90 mm, when the plate was completely covered by the colony).

Mycotoxin	Temperature	UG AF861	UG AF60	UG AF93	UG AF36	UG AF62	UG AF54	UG AF411	UG AF82	UG AF06	UG AF362	UG AF35
AFB2	22°C	ND[a] (0/2)[b]	1742.7, 1785.6[d] (2/2)	352.9, 784.9 (2/2)	50.2 (1/2)	60.3, 83.3 (2/2)	133.6, 193.5 (2/2)	ND (0/2)	ND (0/2)	1611.9, 4964.9 (2/2)	ND (0/2)	365.0, 3728.8 (2/2)
	30°C	<LOQ[c] (1/3)	166±25.8 (3/3)	70.3±56.2 (3/3)	3.2-4.5 (2/3)	40.9±47.7 (3/3)	78.9±52.1 (3/3)	ND (0/3)	4.0-11.7 (2/3)	728.9±526.2 (3/3)	<LOQ (1/3)	624.5±193.7 (3/3)
	37°C	ND (0/3)	5.18 (1/3)	ND (0/3)	ND (0/3)	ND (0/3)	ND (0/3)	ND (0/3)	ND (0/3)	ND (0/3)	ND (0/3)	ND (0/3)
AFB1	22°C	ND (0/2)	14400, 16460 (2/2)	3655.5, 9627.7 (2/2)	598.5 (1/2)	516.7, 823.6 (2/2)	889.7, 1466.4 (2/2)	ND (0/2)	ND (0/2)	17870, 40350 (2/2)	<LOQ (2/2)	3627.2, 31749 (2/2)
	30°C	<LOQ (2/3)	2287.8±308.8 (3/3)	936.4±780.5 (3/3)	35.0±28.5 (3/3)	579.0±660.8 (3/3)	653.4±836.9 (3/3)	<LOQ (2/3)	35.8-160.3 (2/3)	8004.5±1563.4 (3/3)	15.7 (1/3)	6347.2±471.7 (3/3)
	37°C	ND (0/3)	42.1 (1/3)	23.6 (1/3)	ND (0/3)	ND (0/3)	1.37 (1/3)	ND (0/3)	ND (0/3)	12.6 (1/3)	ND (0/3)	ND (0/3)
STERIG	22°C	ND (0/2)	2.4, 2.7 (2/2)	2.7, 5.8 (2/2)	2.0 (1/2)	2.2, 2.6 (2/2)	2.1 (1/2)	ND (0/2)	ND (0/2)	4.5, 21.7 (2/2)	ND (0/2)	4.7, 19.1 (2/2)
	30°C	ND (0/3)	21.0±7.6 (3/3)	3.4-3.8 (2/3)	<LOQ (3/3)	8.8±4.9 (3/3)	3-9.2 (2/3)	ND (0/3)	<LOQ (2/3)	109.7±40.1 (3/3)	<LOQ (1/3)	43.2±13.2 (3/3)
	37°C	ND (0/3)	1.06 (1/3)	0.61 (1/3)	ND (0/3)	ND (0/3)	ND (0/3)	ND (0/3)	ND (0/3)	1.4 (1/3)	ND (0/3)	ND (0/3)
OMST	22°C	ND (0/3)	177.7±44.5 (3/3)	214.5±109.5 (3/3)	64.3, 681.4 (2/3)	156.4±156.2 (3/3)	76.5±62.7 (3/3)	177.9 (1/3)	ND (0/3)	6282±4939.5 (3/3)	6.1, 6.4 (2/3)	677.8±840 (3/3)
	30°C	<LOQ (1/3)	366±126.5 (3/3)	53.7±45.2 (3/3)	41.2±35.9 (3/3)	146.1±64.2 (3/3)	39.1±37.6 (3/3)	<LOQ (2/3)	5.2, 15.7 (2/3)	4674.9±2816.7 (3/3)	125.6 (1/3)	292.1±129.6 (3/3)
	37°C	ND (0/2)	80.7 (1/2)	204.5 (1/2)	ND (0/2)	ND (0/2)	ND (0/2)	ND (0/2)	ND (0/2)	16.5 (1/2)	ND (0/2)	ND (0/2)

[a] Not detected; [b] Number of positives per total number of replicates; [c] Limit of quantification; [d] Both values are presented because of duplicate analysis.

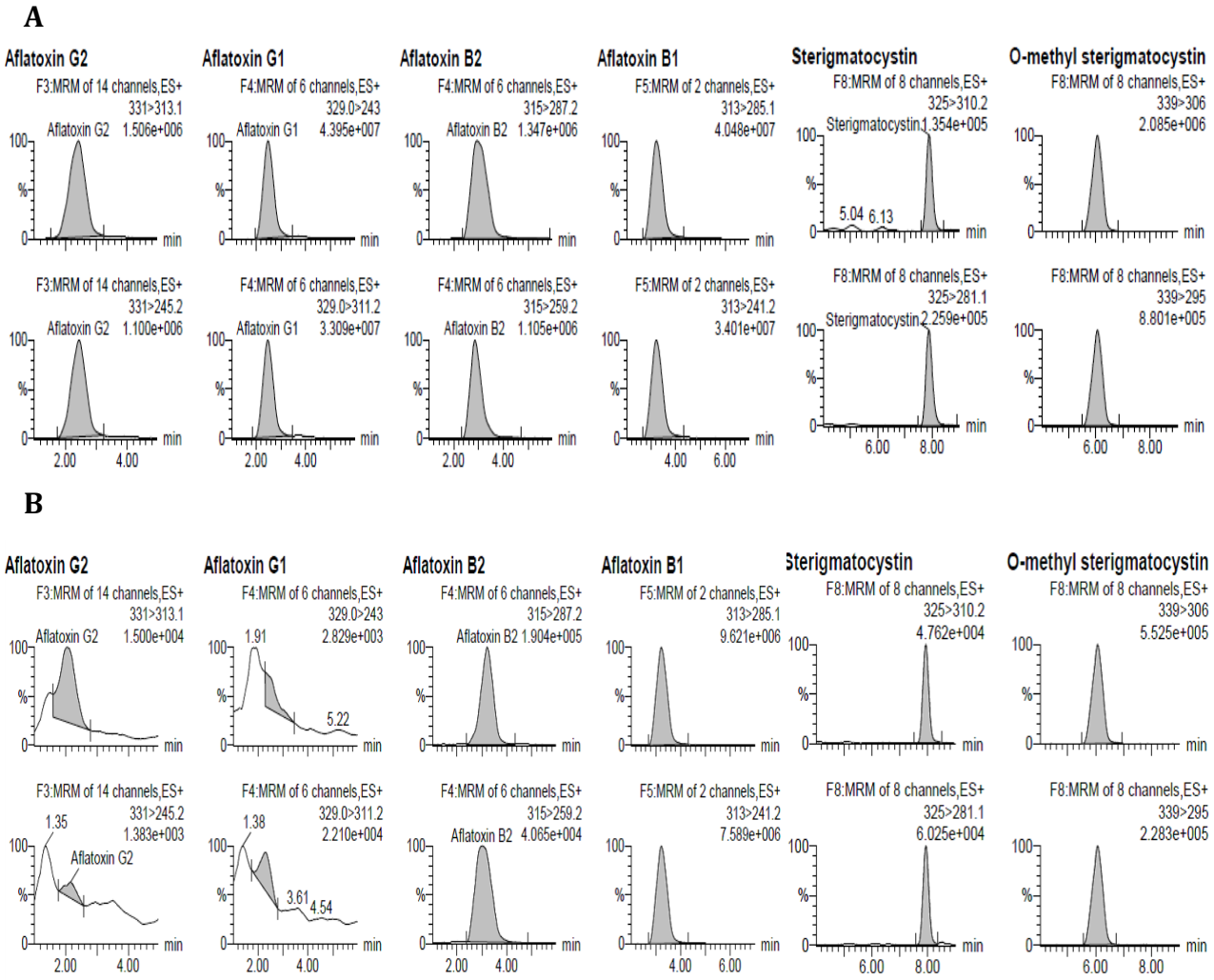

Fig. 7-3. MRM chromatograms of AFG2, AFG1, AFB2, AFB1, STERIG and OMST produced by A) *A. parasiticus* strain UG AP542 and B) *A. flavus* strain UG AF62 in MEA at 30°C. Quantification and confirmation transitions are shown for each metabolite.

7.3.4.2. Toxigenic potential of *A. parasiticus* isolates

All of the *A. parasiticus* isolates produced AFG2, AFB1 and OMST at 30°C (Tables 7-6 and 7-8). At 22°C, 83% of the isolates produced AFBs, STERIG and OMST while only one isolate produced AFGs (Table 7-6). Similar to *A. flavus* species, only two *A. parasiticus* were able to produce mycotoxins at 37°C. Among all the isolates, the highest concentration of aflatoxins (185-6,500 µg/kg), STERIG (59±15 µg/kg) and OMST (821±1260 µg/kg) were produced by the isolate UG AP542 at 30°C (Table 7-7). The isolate UG AP61, AP631 and AP28 produced a high concentrations of B aflatoxins (max 12 mg/kg) and OMST (max. 1170 µg/kg) at 22°C than at 30°C. Other isolates were found to be very low AFG producers in this medium, however they produced considerably higher amount of AFBs, STERIG and OMST. UG AP821 was the weakest mycotoxin producer among all the *A. parasiticus* isolates. According to Rank et al. (2011) the chemical potential of filamentous fungi is highly influenced by the growth conditions, particularly by nutrients. Many different species required different media to produce high levels of mycotoxins. According to Rodrigues et al. (2009), *A.*

parasiticus strains are uniform in their toxigenic ability and usually strongly aflatoxigenic. Other authors have reported that non-toxigenic *A. parasiticus* isolates are extremely rare (Horn et al., 1996; Tran-Dinh et al., 1999). In a study by Vaamonde et al. (2003), only 2 were non-toxigenic of the 30 *A. parasiticus* strains isolated from peanuts.

Table 7-8. Mycotoxin production (mean±SD µg/kg) of different *A. parasiticus* strains isolated from black pepper following the growth in malt extract agar at different temperatures (determined at colony diameter ~80-90 mm, when the plate was completely covered by the colony).

Mycotoxin	Temperature	UG AP821	UG AP631	UG AP61	UG AP542	UG AP462	UG AP28
AFG2	22°C	ND[a] (0/2)[b]	ND (0/2)	ND (0/2)	33.1, 48.3[c] (2/2)	ND (0/2)	ND (0/2)
	30°C	1.4±0.3 (3/3)	2.5, 2.5 (2/3)	10.8±6.9 (3/3)	301.6±155.8 (3/3)	1.6, 3.0 (2/3)	3.2±1.3 (3/3)
	37°C	ND (0/3)	ND (0/3)	ND (0/3)	5.79, 6.36 (2/3)	ND (0/3)	4.6, 5.3 (2/3)
AFG1	22°C	ND (0/2)	ND (0/2)	ND (0/2)	376.6, 447.4 (2/2)	ND (0/2)	ND (0/2)
	30°C	ND (0/3)	9.5 (1/3)	<LOQ[d] (2/3)	185±708.5 (3/3)	<LOQ (1/3)	<LOQ (2/3)
	37°C	ND (0/3)	ND (0/3)	ND (0/3)	5.20 (1/3)	ND (0/3)	ND (0/3)
AFB2	22°C	ND (0/2)	259.6 (1/2)	652.0, 1281.5 (2/2)	44.0, 44.2 (2/2)	7.7, 8.5 (2/2)	165.0, 1007.2 (2/2)
	30°C	ND (0/3)	3.14 (1/3)	529.3±184.0 (3/3)	667.7±246.2 (3/3)	50.1 (1/3)	133.6±85.5 (3/3)
	37°C	ND (0/3)	ND (0/3)	ND (0/3)	3.0, 21.7 (2/3)	ND (0/3)	ND (0/3)
AFB1	22°C	ND (0/2)	2698.5 (1/2)	3716.2-12002.1 (2/2)	475.6, 637.6 (2/2)	35.1, 98.9 (2/2)	1022.3, 8697.8 (2/2)
	30°C	<LOQ (1/3)	33.0-43.9 (2/3)	5403.2±1475.5 (3/3)	5103.3±1359.6 (3/3)	1.2, 653.4 (2/3)	1947.1±1231.6 (3/3)
	37°C	ND (0/3)	ND (0/3)	ND (0/3)	114.3, 307.7 (2/3)	ND (0/3)	ND (0/3)
STERIG	22°C	ND (0/2)	5.6 (1/2)	1.8-23.1 (2/2)	3.7, 4.8 (2/2)	1.9-2.0 (2/2)	4.3, 24.5 (2/2)
	30°C	ND (0/3)	<LOQ (2/3)	49.0±35.7 (3/3)	58.5±15.0 (3/3)	<LOQ (1/3)	15.4±5.4 (3/3)
	37°C	ND (0/3)	ND (0/3)	ND (0/3)	0.42, 0.80 (2/3)	ND (0/3)	ND (0/3)
OMST	22°C	ND (0/3)	110.6, 188.9 (2/3)	725.1±548.9 (3/3)	49.4±2.5 (3/3)	20.0±17.7 (3/3)	204.8±265.0 (3/3)
	30°C	0.3, 2.7 (2/3)	0.9, 10.2 (2/3)	251.1±127.4 (3/3)	1096.2±239.9 (3/3)	1.7, 17.6 (2/3)	195.7±79.3 (3/3)
	37°C	ND (0/2)	ND (0/2)	ND (0/2)	33.1 (1/2)	ND (0/2)	ND (0/2)

[a]Not detected; [b]Number of positives per total number of replicate; [c]Both values are presented because of duplicate experiments or two positive replicates; [d]Limit of quantification.

Aflatoxin G production in malt extract agar was found to be much lower compared to the AFB production of most of the isolates. It might be worth to mention here also that the production pathway of AFB2 and AFG2 is different from that of AFB1 and AFG1. The critical branch point leading to the

formation of either AFB1/AFG1 or AFB2/AFG2 is versicolorin B (Fig. 1-5). Production of OMST is only necessary for AFB1 and AFG1 production, while transformation of versicolorin B to dihydro-O-methyl sterigmatocystin (DHOMST) is necessary for AFB2 and AFG2 production (Yu, 2012; Cleveland et al., 2009). According to Georgianna and Payne, (2009) the regulation of STERIG and especially aflatoxin production by *Aspergillus* generally require simple sugars, low pH, reduced nitrogen source and mild oxidative stress. Recent studies have even shown that when *A. flavus* was grown in peptone-containing media, higher initial spore densities inhibited aflatoxin biosynthesis, but promoted mycelial growth (Yan et al., 2012). However, it is still hard to say that this could be the reason for low AFG production observed in MEA, since they still produced high level of B aflatoxins. May be genetically those isolates are low AFG producers. More research is necessary to elucidate this in detail. Moreover, aflatoxigenic ability in *A. flavus* seems to have a very unstable character. Its adaptation to carbon rich environments of certain agricultural commodities may be involved in mutations that in turn make it loose its toxigenic potential (Rodrigues et al., 2009). Substrate composition could have a significant influence on the toxigenic potential of the same fungal isolate even at similar growth conditions. Thus, it is very essential to report the toxigenicity of the fungal isolate together with the growth substrate and growth conditions.

7.3.5. Correlation between secondary metabolite production among different isolates

The production of mycotoxins was found to be very much strain specific and highly temperature dependent among the *Aspergillus* isolates of black peppers. As mentioned, significant variability in their production was also found even between replicates of the same isolate at identical growth conditions, which is rather challenging to explain. Nevertheless, the pooled data set (using the mycotoxin production data of all the replicates of both *A. flavus* and *A. parasiticus* species) shows some notable correlations in mycotoxin production between the different isolates (Fig. 7-4).

A non-linear correlation (expressed as coefficient of determination R^2) was found between STERIG-AFB1 (0.81) and OMST-AFB1 (0.79), while a linear correlation existed between OMST-STERIG (0.80) and AFB1-AFB2 (0.87) production. In fact, considering the biosynthesis, STERIG is converted to OMST to produce AFB1 thus high correlation between these metabolites could be conceivable. However, this higher correlation between AFB1 and AFB2 production has not been previously reported which makes it an important observation in view of their biosynthesis. Though both AFB1 and AFB2 have similar precursors in the initial stages of their biosynthesis, however, conversion of versicolorin B to versicolorin A in later stages was necessary for bio-transformation to AFB2 (Fig. 1-5). However, in naturally contaminated samples generally AFB1 was found to occur more frequently and at higher concentration than AFB2. The growth media used in this study might have played a significant role to obtain this high correlation between these two mycotoxins. As previously mentioned, the simple sugars and peptone with complex sugars (lactose, mannose, xylose, galactose)

have shown different effects in fungal growth and mycotoxin production (Georgianna and Payne, 2009; Calvo et al., 2002). Because of the small number of isolates and low production of AFG toxins, possible correlations of AFGs with other mycotoxins production was not investigated.

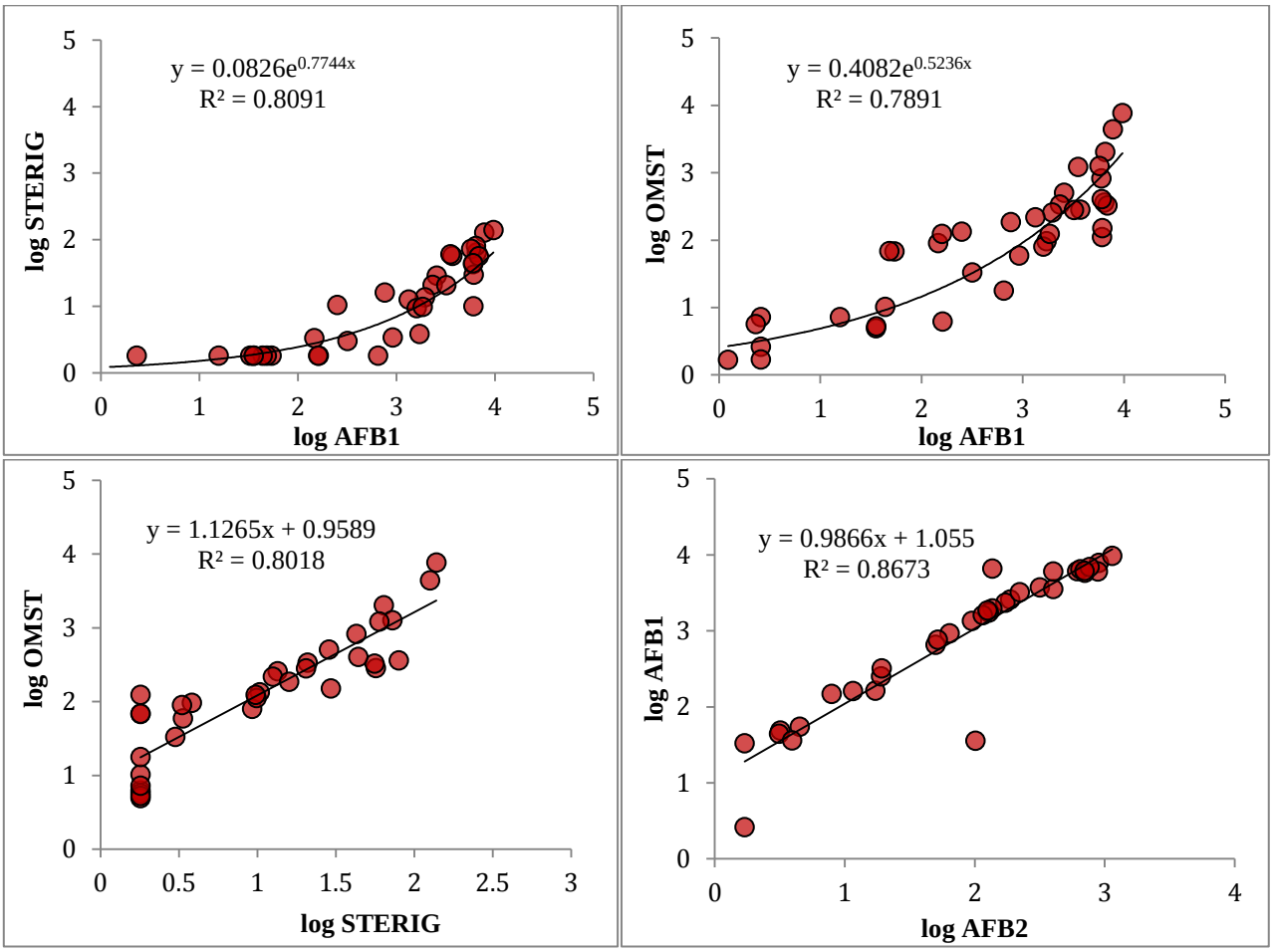

Fig. 7-4. Correlation between the logarithmic concentrations of secondary metabolites, sterigmatocystin (STERIG), O-methyl sterigmatocystin (OMST), aflatoxin B2 (AFB2) and aflatoxin B1 (AFB1) produced by *A. flavus* (n=11) and *A. parasiticus* (n=6) isolates.

Moreover, it was interesting to note that OMST was detected whenever there was AFB1 production; a 100% association in occurrence between OMST and AFB1 was found at 30°C for both the fungal species, *A. flavus* and *A. parasiticus*. Besides, when there was high AFB1 production generally high OMST (and low STERIG) was produced by the isolates of both species regardless of the temperature. Hence, production of OMST (rather than STERIG) could be used as an indicator for the prediction of AFB1 production by *A. flavus* and/or *A. parasiticus* species. Moreover, the results showed that not all the OMST produced was bio-transformed to AFB1. Considering the whole dataset, STERIG (max 109.7 µg/kg) concentration was found to be very low compared to the OMST (10.9 mg/kg) or AFB1 (40 mg/kg) concentration for all the isolates of both *A. flavus* and *A. parasiticus* (Tables 7-6 and 7-7). This could be probably due to the rapid bio-transformation of STERIG to OMST (Rank et al., 2011) necessary for AFB1 production.

Aflatoxin biosynthesis requires a complex regulatory mechanism orchestrated by the pathway-specific regulatory genes, *aflR* and to a lesser extent *aflS* (Amare and Keller, 2014). Moreover, *aflQ* is the only gene involved in transforming OMST to AFB1, a unique step in aflatoxigenic species (Rodrigues et al., 2009) (Fig. 1-5). Thus, it appears that the toxigenic potential of any fungal isolate is mainly about their gene expression in different growth substrates and conditions (more information on aflatoxin biosynthesis and their regulation is provided in **Chapter 1**). A higher number of isolates of different fungal species need to be studied on their mycotoxigenic potentiality as well on their toxigenic stability in different growth medium and conditions. This could help to confront their complexity in secondary metabolism and to define possible measures to control the production of these toxic secondary metabolites in food and feed products, harmful to human and animal health.

7.4. CONCLUSIONS

An LC-MS/MS based multi-mycotoxin method was developed to determine the production of mycotoxins by pure fungal cultures in malt extract agar and was successfully validated. The method was applied to assess the toxigenicity of *A. flavus* and *A. parasiticus* species isolated from black pepper. Mycotoxin production was very much temperature dependent, as well as strain specific for both species. There was no correlation between the growth rate and any of the secondary metabolite production of both these fungal species. However, notable correlations were found between the concentrations of the different metabolites in the substrate used. A strong correlation between AFB2 and AFB1 concentration was observed despite differences in their biosynthetic pathways in later stages. Low STERIG level, high OMST and AFB1 concentration in this substrate suggest that OMST could be used as a predictor for AFB1 production in both fungal species. The developed method may be of great importance for chemotaxonomic research and to study the conditions which could induce or suppress the complex secondary metabolism of various pure fungal isolates.

Aspergillus flavus and *A. parasiticus* fungal isolates selected from this toxigenicity study were used in the development of predicitve fungal growth models of these species and to study their mycotoxin production potential at different growth conditions on peppercorns as described in the next chapter (**Chapter 8)**.

CHAPTER

8

MYCOTOXIN PRODUCTION AND PREDICTIVE MODELLING KINETICS ON THE GROWTH OF *Aspergillus flavus* AND *Aspergillus parasiticus* IN WHOLE BLACK PEPPERCORNS

CHAPTER 8: MYCOTOXIN PRODUCTION AND PREDICTIVE MODELLING KINETICS ON THE GROWTH OF *Aspergillus flavus* AND *Aspergillus parasiticus* IN WHOLE BLACK PEPPERCORNS

Summary

The growth and mycotoxin production of an *Aspergillus parasiticus* and three *A. flavus* isolates were studied in whole black peppercorns (*Piper nigrum* L.) using a full factorial design with seven water activity (a_w) (0.826-0.984) levels and three temperatures (22, 30 and 37°C). Growth rates and lag phases were estimated using linear regression. Diverse secondary models were assessed for their ability to describe the radial growth rate as a function of individual and combined effect of a_w and temperature. Optimum radial growth rate ranged from 0.75±0.04-2.65±0.02 mm/day for *A. flavus* and 1.77±0.1-2.5±0.1 mm/day for *A. parasiticus* based on the Rosso square root cardinal estimations. Despite the growth failure of some isolates at marginal conditions, all the studied models showed good performance to predict the growth rates. The bias factors (0.70-1.01), accuracy factors (1.01-1.41) and root mean square error (0.019-0.280) show that the examined models are conservative predictors of the colony growth rate of both fungal species in black peppers. The Rosso square root cardinal model can be recommended to describe the individual a_w effect while the extended Gibson model was the best model for describing the combined effect of a_w and temperature on the growth rate of both fungal species in peppercorns. Temperature optimum ranged from 29 to 33°C, while a_w optimum was 0.93-0.96 as estimated by multi-factorial cardinal model for both species. Following the growth study, production of mycotoxins (aflatoxins B1, B2, G1, G2, sterigmatocystin and O-methyl sterigmatocystin (OMST)) were quantified using LC-MS/MS. Very small quantities of AFB1 (<LOQ-9.1 µg/kg) were produced only by *A. parasiticus*. OMST was not produced in any growth conditions by both species. Sterigmatocysin (<LOQ-76.7 µg/kg) was the dominant mycotoxin found. High inter- and intra-species variability in mycotoxin production restricted the modelling of mycotoxin production in black pepper. The estimated minimum temperature and a_w for *A. flavus* growth were 6.7-18.2°C and 0.74-0.83, respectively, and for *A. parasiticus* the corresponding values were 12.5°C and 0.7-0.8, hence, achieving these conditions should be considered during storage to prevent the growth of these mycotoxigenic fungal species in black peppercorns.

Keywords: *Black pepper, Aspergillus flavus, Aspergillus parasiticus, Growth rate, Predictive modelling, LC-MS/MS.*

Relevant publication: Yogendrarajah, P., Vermeulen, A., Jacxsens, L., Mavromichali, E., De Saeger, S., De Meulenaer, B. and Devlieghere, F. (2014). Mycotoxin production and predictive modelling kinetics on the growth of *Aspergillus flavus* and *Aspergillus parasiticus* isolates in whole black peppercorns (*Piper nigrum* L.). In preparation.

8.1. INTRODUCTION

Fungal spoilage and mycotoxin contamination of foodstuff are worldwide problems causing large economic losses and a serious risk to public health. As highlighted in previous chapters (**Chapter 1-3**) black peppers are often contaminated with several toxic moulds and mycotoxins. The most important moulds are the *Aspergillus* spp., *A. flavus* and *A. parasiticus,* as reported from different countries. In addition to the fungal infestation, black peppers have been frequently reported to be contaminated with aflatoxins and OTA (Yogendrarajah et al., 2014a; **Chapter 3**) at various levels of concentrations. Thus, there is a need to control mould growth and consequently mycotoxin contamination in black pepper.

In order to improve the microbiological quality and safety of food, tools allowing the prediction of fungal growth are essential (Dantigny et al., 2005a; Dagnas and Membré, 2013). The imperious need for characterizing the effects of factors that govern fungal growth during pre- or post-harvest stages triggered the interest in the application of mathematical approaches to describe and predict the fungal response to different growth factors. Several probability, mechanistic, semi-mechanistic, empirical and thermal death models have been developed for a variety of toxigenic and spoilage fungal species (Gibson et al., 1994; Rosso and Robinson, 2001; Sautour et al., 2002; Garcia et al., 2009). A growing number of studies are now available dealing with the modelling approach to predict fungal growth and mycotoxin production in different food products. In the case of potent aflatoxin producers, *A. flavus* and/or *A. parasiticus*, very limited number of studies are available reporting the effect of water activity (a_w) and temperature on fungal growth that has been performed directly in real food matrices like, yellow dent corn (Samapundo et al., 2007c), maize grain (Garcia et al., 2013), pistachio nuts (Marín et al., 2012), polished and brown rice (Mousa et al., 2013). Though, it has been widely accepted that prevention of fungal growth will eventually prevent the accumulation of mycotoxins, predictive mycology is still way behind (models are still need to be developed for diverse fungal species on various food and feed matrices). Studies are yet available to predict these fungal growth and mycotoxin production in one of the economically significant spice, black pepper.

Hence, the aim of the present study was to, (i) quantify the individual and combined effect of a_w and temperature on the radial growth rate and mycotoxin production of different strains of *A. flavus* and *A. parasiticus* in black peppercorns, (ii) develop mathematical models describing the effect of a_w and temperature on growth kinetics and assess their performance on prediction and (iii) validate the models using independently derived data.

8.2. Materials and methodology

8.2.1. Chemicals, reagents and mycotoxin standards

Anhydrous magnesium sulphate was purchased from Sigma-Aldrich, Steinheim, Germany. Other chemicals and reagents used for LC-MS, MeCN and sodium chloride used for sample extraction were of analytical grade, same as described in **Chapter 2 (section 2.2.1).**

The mycotoxin reference standards of aflatoxin B1 (AFB1), aflatoxin B2 (AFB2), aflatoxin G1 (AFG1), aflatoxin G2 (AFG2) and sterigmatocystin (STERIG) were the same purchased from Sigma-Aldrich (Bornem, Belgium). O-methyl sterigmatocystin (OMST) was supplied by Chromadex (California, USA). From the individual stock solutions, the working solutions were prepared by diluting in MeOH. A standard mixture of mycotoxins were prepared at the following concentrations, AFB1, AFB2, AFG1, AFG2, OMST (0.5 µg/mL) and STERIG (0.625 µg/mL).

8.2.2. Fungal isolates and inoculum preparation

Three toxigenic *A. flavus* (UG AF06, UG AF35, UG AF60) strains and one *A. parasiticus* (UG AF542) strain were selected for this study. These strains were isolated from black pepper (*Piper nigrum* L.*)* samples collected from Sri Lanka (**Chapter 3**). Species level identification (by molecular techniques) of these strains were confirmed at MUCL (Laboratoire de Mycologie, Louvain-la-Neuve, Belgium). Since, the ratio of aflatoxin B producers (*A. flavus*) to B+G (*A. parasiticus*) producers among the 38 toxigenic strains was ~3:1 (**Chapter 7**), it was decided to work on three *A. flavus* and one *A. parasiticus* to be representative of the total toxigenic isolates. These isolates have been previously characterized to be heavy producers of several secondary metabolites in malt extract agar. *A. flavus* produced AFB2, AFB1, STERIG and OMST while *A. parasiticus* produced AFG2, AFG1, AFB2, AFB1, STERIG and OMST (**Chapter 7**). The UG AP542 was the only *A. parasiticus* to produce high concentration of the four aflatoxins in MEA, hence, it was selected to study further. Spore solutions (10^6 CFU)/mL of each isolate were prepared from a 10 days old cultures in malt extract agar as described in **Chapter 7 (section 7.2.3).**

8.2.3. Experimental design

The experimental design corresponded to a full factorial design with two main factors, temperature and water activity (a_w). The incubation temperatures studied were 22, 30 and 37°C, while the seven a_w levels investigated were 0.826, 0.857, 0.892, 0.921, 0.936, 0.961 and 0.984. Six plates per condition, per isolate were prepared.

8.2.4. Preparation of growth substrate, inoculation and incubation

The growth and mycotoxin production of *A. flavus* and *A. parasiticus* strains were studied in whole black peppercorns of Sri Lankan origin. To ensure no fungal infection or mycotoxin contamination, the peppercorns were sterilized at 25 kGy by gamma irradiation at Synergy Health (Ede, Netherlands). Samples were stored aseptically in the refrigerator until further use.

The a_w of black peppercorns was adjusted asceptically to the desired a_w levels by adding appropriate amounts of sterile water using the moisture sorption isotherms developed for each temperature (**Chapter 6**). To ensure uniform adsorption of water the peppercorns were equilibrated at the respective experimental temperatures for two days. The containers were regularly shaken to confirm identical adsorption of water by the peppercorns. The actual a_w of the peppercorns was verified using the a_w meter (Novasina LabMaster, Lachen SZ, Switzerland). Initial a_w and moisture content of the peppercorns were 0.60±0.06 and 12.5±0.7 g/100g dry matter, respectively.

The rehydrated pepper corns were aseptically transferred to a 90 mm Petri plate and arranged to form a single compact layer. Using a sterile cork borer, a 5 mm diameter agar disc (based on Mousa et al., 2011) was centrally positioned in each plate and inoculated with 10 µL of the prepared spore solution (10^6 CFU/mL). All the six replicates containing inoculated peppercorns of the same a_w were carefully wrapped with parafilm and placed on a plastic mesh with support in a closed container. Each container was filled with glycerol-water solution (200 mL) of the same a_w as that of peppers to ensure fixed equilibrium relative humidity (ERH) during the incubation period. The glycerol-water solution of a particular a_w was prepared based on the glycerol-a_w curves developed for that particular temperature as explained below. The containers were incubated at 22, 30 and 37°C. An extra plate with peppercorns of the same a_w was placed as well in the same container and a_w was measured to confirm the constant ERH throughout the incubation period.

For the development of glycerol a_w-curve, different concentrations of glycerol solutions between 10 to 60 g/100 mL were prepared in water. The a_w of these solutions was measured at three temperatures and second order polynomial curves specific for each temperature were developed by plotting a_w (y-axis) against glycerol concentrations (x-axis). Following quadratic equations were derived (Eq. 8-1, 8-2 and 8-3);

For 25°C $y=-3E-07x^2 – 0.002x + 0.09983;\ R^2\ 0.9983$ Eq. 8-1

For 30°C $y=-1E-07x^2 – 0.0003x + 0.09973;\ R^2\ 0.9978$ Eq. 8-2

For 37°C $y=5E-09x^2 – 0.0003x + 1.0063;\ R^2\ 0.9872$ Eq. 8-3

8.2.5. Growth assessment

All the six plates from each growth condition were assessed for fungal growth on a daily basis or at regular time intervals. The colony growth of each isolate was established by diametric measurements at orthogonal directions (x, y) using an electronic digital caliper. The mean value of the two diameters was calculated and used in the modelling after correcting for its growth in agar disc. Growth measurements were carried out until the colony reached the edge of the plate (complete colonization) or when growth stopped. At the same time initiation of the sporulation process was also recorded.

8.2.6. Mathematical models

8.2.6.1. Primary modelling

Growth curves were developed by plotting the mean colony diameters (mm) of each of the six cultured plates against incubation time (days) to estimate the growth rate. Maximal radial growth rate (μ_{max}, mm/day) was finally estimated using the lineal model/linear regression of the growth curve. The lag phase or the time to visible growth (λ, days) was estimated through the interception between the regression line and the x-axis.

8.2.6.2. Secondary modelling

The estimates of the μ_{max} were fitted by several secondary models to describe the individual and combined effect of temperature and a_w on growth rate of both fungal species in black peppercorns. To describe the individual effect on the growth response of the different fungal strains, four secondary models were evaluated as a function of a_w at different temperatures.

First, the μ_{max} was modelled as a function of a_w for each temperature level by the Ratkowsky square-root model extended for $a_{w,max}$ (Tassou et al., 2007). An explicit version of the model is the following:

$$\sqrt{\mu_{max}} = b(a_w - a_{w,min})\{1 - \exp[c(a_w - a_{w,max})]\} \quad \text{Eq. 8-4}$$

where b, c, $a_{w,min}$, and $a_{w,max}$ are estimated constants, $a_{w,min}$ and $a_{w,max}$ correspond to the values of a_w below and above which no growth occurs (Garcia et al., 2009). An additional advantage of this model is the fact that it includes biologically significant parameters, giving more insight into the behaviour of the strains (Pitt, 1993).

The Rosso cardinal model was the second approach followed to study the effect of the entire range of a_w levels on the growth of the fungi. The advantage of this model is also that all parameters have a physiological meaning which can facilitate the incorporation of important cell biological mechanisms

into the model (Garcia et al., 2009; Rosso and Robinson, 2001). The model is based on the following equation, Eq. 8-5:

$$\mu_{max} = \frac{\mu_{opt}(a_w - a_{w,max})\,(a_w - a_{w,min})^2}{(a_{w,opt} - a_{w,min})\,(a_{w,opt} - a_{w,min})\,(a_w - a_{w,opt}) - (a_{w,opt} - a_{w,max})\,(a_{w,opt} + a_{w,min} - 2a_w)} \quad \text{Eq. 8-5}$$

where $a_{w,opt}$ is the value of a_w at which μ_{max} is equal to its optimal value (μ_{opt}). $a_{w,min}$, $a_{w,max}$ are the minimum and maximum a_w which allows fungal growth. In addition to this established Rosso cardinal model, equation using the square root of μ_{max} was also evaluated as a third approach. Square root transformation was introduced in this model to stabilize the variability in the radial growth rate values as suggested previously (Dantigny and Bensoussan, 2008).

Fourthly, the Gibson model which was developed specifically for moulds was applied. Gibson et al., (1994) applied the following transformation of a_w (Eq. 8-6) for better hyperbolic fitting and the radial growth rate variance was stabilized using the natural logarithm.

$$b_w = \sqrt{1 - a_w} \quad \text{Eq. 8-6}$$

Therefore, the model expression of the maximum colony growth rate takes the following form (Eq. 8-7).

$$\ln\mu_{max} = a_0 + a_1\sqrt{1 - a_w} + a_2\,(1 - a_w) \quad \text{Eq. 8-7}$$

where a_0, a_1, a_2 are constants to be estimated by non-linear regression.

To explain the combined effect of a_w and temperature on the fungal growth rate, additionally four secondary models were evaluated. The second order-polynomial regression model (general polynomial) was applied to evaluate the combined effect of a_w and temperature on fungal μ_{max} using the following equation (Eq. 8-8).

$$\sqrt{\mu_{max}} = a_0 + a_1.a_w + a_2.a_w^2 + a_3.T + a_4.T^2 + a_5.T.a_w \quad \text{Eq. 8-8}$$

where a_0 to a_5 are constants and T, temperature in degree Celsius (°C). Square root transformation of the μ_{max} was introduced in this model for the above said reasons.

The Gibson model (Gibson and Hocking, 1997) was extended for temperature and a second order response surface model was developed (Samapundo et al., 2007c; Tassou et al., 2008). An explicit version of the model is the following (Eq. 8-9):

$$\sqrt{\mu_{max}} = a_0 + a_1.b_w + a_2.b_w^2 + a_3.T + a_4.T^2 + a_5.T.b_w \quad \text{Eq. 8-9}$$

where a_0 to a_5 are the model parameters to be estimated by linear regression using step-wise selection. a_w was replaced by b_w in this equation.

Moreover, the linear Arrhenius-Davey model extended by Panagou et al. (2003) including a_w was also used to model the radial growth data. The model is based on the following equation (Eq. 8-10):

$$\sqrt{\mu_{max}} = a_0 + a_1.a_w + a_2.a_w^2 + {}^{a_3}/_{T} + {}^{a_4}/_{T^2} \quad \text{Eq. 8-10}$$

where T is the temperature and a_0, a_1, a_2, a_3 and a_4 are constants to be determined. Although the model is entirely empirical, it is one of the most studied modifications of the Arrhenius equation to describe the effect of temperature on microbial growth (Ross & Dalgaard, 2003). This model was successfully used as a predictor of the lag phase duration of *A. flavus* and *A. parasiticus* on corn (Samapundo et al., 2007c).

Finally, the estimates of the μ_{max} were further fitted to the multi-cardinal secondary model developed based on the previous cardinal models (Gamma concept) (Rosso et al., 1993; Rosso and Robinson, 2001; Marín et al., 2012; Sautour et al., 2001a). The concept is described by the following equations (Eq. 8-11, 12 & 13).

$$\mu_{max}(T, a_w) = \mu_{opt}.\tau(T).\rho(a_w) \quad \text{Eq. 8-11}$$

where,

$$\tau(T) = \left(\frac{(T-T_{min})^2.(T-T_{max})}{(T_{opt}-T_{min}).[(T_{opt}-T_{min})(T-T_{opt})-(T_{opt}-T_{max})(T_{opt}+T_{min}-2T)]}\right) \quad \text{Eq. 8-12}$$

and

$$\rho(a_w) = \left(\frac{(a_w-a_{w,min})^2.(a_w-1)}{(a_{w,opt}-a_{w,min}).[(a_{w,opt}-a_{w,min})(a_w-a_{w,opt})-(a_{w,opt}-1)(a_{w,opt}+a_{w,min}-2a_w)]}\right) \quad \text{Eq. 8-13}$$

The terms T_{min}, T_{max}, $a_{w,min}$, $a_{w,max}$ correspond to the values of temperature and a_w respectively, below or above which no mould growth occurs. Moreover, the T_{opt} and $a_{w,opt}$ are the values of temperature and a_w at which the μ_{max} is at its optimal value $\mu_{opt.}$

8.2.7. Statistical analysis and model evaluation

Univariate analysis of variance of the general linear model was used to analyze the influence of temperature and a_w on fungal growth rate. Both main and interaction effect of these factors on growth rate were statistically analyzed. The statistical significance was set at $P \leq 0.05$, unless otherwise specified. SPSS statistics (IBM®, Version 22) was used to estimate the parameters of the secondary growth models.

The coefficient of determination (R^2 general or 'adjusted') and root mean square error (RMSE) were the statistical indices used to assess the fit of the models. RMSE was used to measure of the residual variability between predicted and the experimental values of the dependent variable, growth rate. The

equation used to estimate RMSE was based on the sample standard error of the differences between the predicted and experimental values as shown below (Eq. 8-14).

$$\mathrm{RMSE} = \sqrt{\frac{\sum(\mu_{max,pred} - \mu_{max,exp})^2}{n}} \quad \text{Eq. 8-14}$$

where $\mu_{max,pred}$ is the maximum colony growth rate predicted by each model, $\mu_{max,exp}$ is the maximum colony growth rate obtained by the experiment and n is the number of experimental points.

8.2.8. Model validation

The models were validated using the data obtained from repeated experiments on black peppercorns at two temperatures (30 and 37°C) employed for model development and at two different a_w levels, 0.88 and 0.91. As proposed by Ross (1996), performance of the developed models was assessed using the mathematical indices, bias factor (B_f) (Eq. 8-15), accuracy factor (A_f) (Eq. 8-16), RMSE and standard error of prediction (SEP) (Eq. 8-17). SEP is a relative percentage that does not depend on the magnitude of dataset values.

$$B_f = 10^{(\Sigma(\mu_{max,obs} - \mu_{,max,pred})/n)} \quad \text{Eq. 8-15}$$

$$A_f = 10^{(\Sigma|\log(\mu_{max,obs} - \mu_{max,pred}|/n)} \quad \text{Eq. 8-16}$$

$$\mathrm{SEP}\ (\%) = \frac{100}{X_{\mu max,obs}} \sqrt{\frac{\sum(\mu_{max,obs} - \mu_{max,pred})^2}{n}} \quad \text{Eq. 8-17}$$

where, $X_{\mu max,obs}$ is the mean of the observed growth rate, $\mu_{max,obs}$ maximum colony growth rate obtained by the validation experiment, $\mu_{max,pred}$ and n are same as explained above for Eq. 8-14.

8.2.9. Analysis of mycotoxins following growth

Following the growth study of different isolates in black pepper, the samples were analysed to determine the production of aflatoxins (G2, G1, B2, B1), sterigmatocystin and O-methyl sterigmatocystin. Mycotoxin analysis was performed when the fungal colony diameter was ~80-90 mm (colony covered the whole plate) or when growth stopped.

8.2.9.1. Sample preparation

The whole content of the Petri plate (black peppercorns together with the fungal biomass) were finely ground using a motor and pestle prior to analysis (too small quantity of the sample restricted the use of a mechnaical grinder for grinding the samples). Mycotoxins produced in black peppers were

extracted using the method developed in-house as described in **Chapter 2 (section 2.2.4.** (Yogendrarajah et al., 2013).

8.2.9.2. Instrumentation and conditions

The instrumentation (LC-MS/MS) and the conditions for mycotoxins analysis were the same as previously described in **Chapter 2 (section 2.2.5).**

8.3. RESULTS AND DISCUSSION

8.3.1. Primary modelling: growth rate and lag phase

The growth of the selected *A. parasiticus* (UG AP542) and *A. flavus* (UG AF06, UG AF35 and UG AF60) isolates in black pepper followed, in general a lag-linear curve with few exceptions mainly under marginal conditions. The estimated maximum colony growth rates (μ_{max}, mm/day) and lag phase durations (λ, days) of all the isolates at all the experimental conditions are shown in Table 8-1. At optimum growth conditions, the growth was very reproducible and the variability between replicates was very small (Fig. 8-1; Table 8-1).

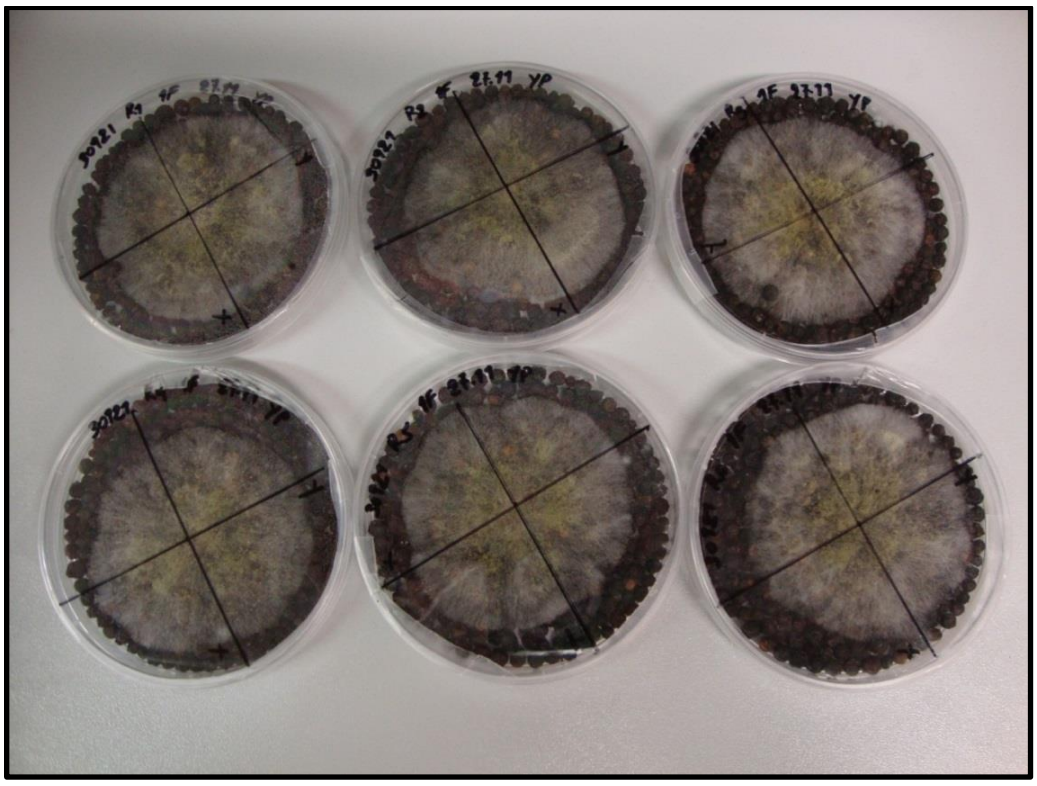

Fig. 8-1. *Aspergillus flavus* isolate UG AF06 grown in whole black peppercorns at a_w 0.921 showing good reproducibility among all the replicates (n=6) after six days of incubation at 30°C.

In this study, only growth rate was chosen for secondary modelling as it is the mostly used parameter in fungal modelling. Generally, the variability observed with the growth rate was smaller than that of

lag phase, even at marginal growth conditions (Table 8-1). Moreover, there seem to be no systemic trend in lag phases with some strains (Eg., UG AF35 at 22 and 37°C) compared to growth rate. The initial assumption on probable influence of agar plug on lag phase also to some extent hindered the use of lag phase in modelling (discussed in detail below). According to (Marín et al., 2008a) more than 75% of the publications using growth rate for secondary modelling and it complements the lag phase data. However, the models based on lag phase could be useful to estimate the time to visible growth (3 mm). Generally, it is considered that the lag phase and the time for visible growth should have similar values or the latter might be slightly higher. According to Dantigny et al., (2002), lag phase coincides with the completion of mould germination.

In most cases, an increase in incubation temperature from 22 to 30°C resulted in an increase in the colony growth rate and a decrease in the lag phase. Further increase in temperature from 30 to 37°C generally resulted in a decrease in the colony growth rate (except for UG AF60) and an increase in the lag phase duration. The growth curves of *A. parasiticus* (UG AP542) and *A. flavus* (UG AF06) at different temperatures are shown in Fig. 8-2. The strain UG AP542 failed to grow at the two extreme conditions experimented in this study, 22°C-0.984 a_w and 37°C-0.826 a_w. The *A. flavus* isolate UG AF06 only failed to grow at 37°C-0.826 a_w. However, the *A. flavus* UG AF35 was found to grow at all the conditions even at the extremes. Moreover, UG AF60 failed to grow at rather high a_w conditions at all the temperatures and also at 37°C-0.826 a_w. Generally, linear growth was observed at intermediate a_w values with faster growth rates and shorter lag phases, while an extended lag phase was observed under extreme low or extreme high a_w levels. Maximum growth rate was observed at intermediate a_w (0.892-0.936) levels; 5.38-6.92 mm/day for UG AP542 at 30°C and 6.07-7.25 mm/day at 37°C for UG AF06.

It should be mentioned that in some cases, especially at 22°C in combination with very high or very low a_w levels, the incubation time was very long. For instance, it took almost three months for the isolate UG AF06 to fully cover the plate at 22°C-0.826 a_w, with a growth rate of 0.70±0.14 mm/day. There was a significant influence of temperature, a_w and the combination on the radial growth rate and lag phases of both *A. parasiticus* and *A. flavus* isolates in black pepper ($p<0.001$). In some cases, considerable variability in growth was found at extremely low and extremely high a_w conditions. Marín et al. (2009) reported a similar behaviour with increased variability in colony growth of *A. flavus* at low a_w treatments of red chilli powder while Samapundo et al. (2007c) reported high variability at high a_w conditions (>0.99) in corn. The low growth potential at high a_w conditions could be explained by the xerophilic nature of these *Aspergillus* isolates.

It should be mentioned here that the a_w of the agar plug was not adjusted to that of the peppercorns. However, our initial studies have shown that the mould isolates used in this study started to grow in peppercorns in one or two days following their inoculation directly on peppercorns at optimal growth conditions (direct inoculation on peppercorns was not performed since the spore solution flow below the peppercorns and lead to higher variability on growth). The 5 mm diameter of the plug was subtracted (corrected for the growth in agar) in the growth measurements to calculate the growth rate in peppers. Possible argument is that the moulds might use the nutrients absorbed from the agar to grow in peppercorns for some extent. However, this may not be completely true since these isolates also did not show growth at higher a_w of the peppercorns despite its growth in the agar plug. If there was such influence of carryover of nutrients at least it could have showed some growth when incubated at 22°C/0.984 in peppercorns as soon it has grown in agar (no growth was observed for UG AP542 and UG AF06). Considerably longer lag phases (8.5-17 days) were observed for the two strains (UG AF06 and UG AF35) at 22°C/0.984. Growth in pepper at 22°C/0.961 was also not observed immediately after it has grown in MEA but, it took some time to grow in peppers depending on the type of strain (Fig. 8-2). Hence, it could be presumed that the moulds were probably using the nutrients from the peppercorns for their growth since growth was initiated in peppercorns at different days (different lag phaes observed). Moreover, growth in the agar also highly dependent on the strain as well as the incubation temperature (**Chapter 7**).

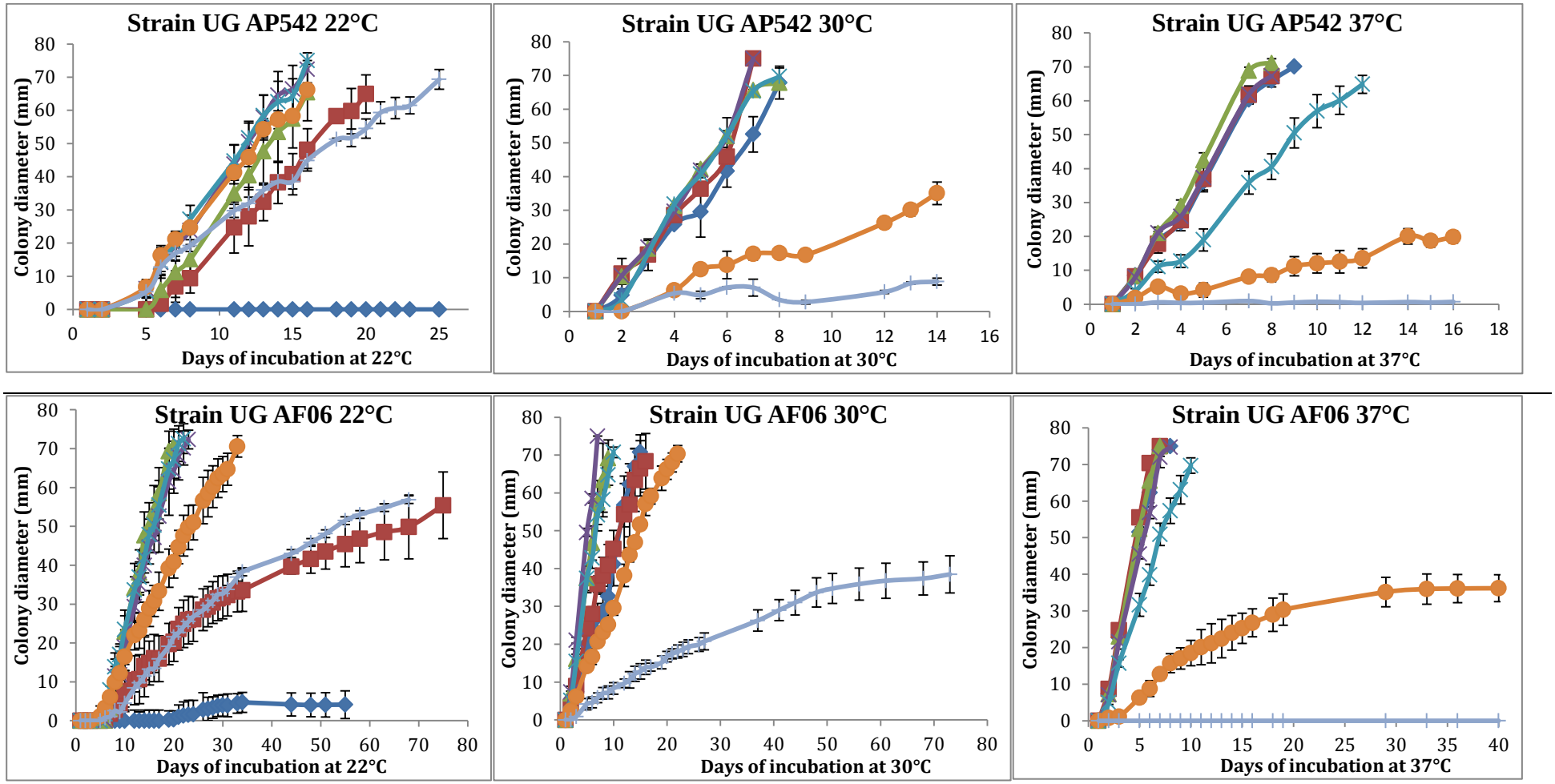

Fig. 8-2. Growth curves of *A. parasiticus* (UG AP542) and *A. flavus* (UG AF06) isolates in whole black peppercorns at 22, 30 and 37°C (0.984 a_w, 0.961 a_w, 0.936 a_w, 0.921 a_w, 0.892 a_w, 0.857 a_w, 0.826 a_w).

Table 8-1. Maximum radial growth rates (μ_{max}) (mean±SD, mm/day) and lag phases (λ) (mean±SD, days) of *A. parasiticus* (UG AP542) *A. flavus* (UG AF06, UG AF35 and UG AF60) and strains in whole black peppercorns.

Temperature (°C)	a_w	UG AP542		UG AF06		UG AF35		UG AF60	
		μ_{max}	λ	μ_{max}	λ	μ_{max}	λ	μ_{max}	λ
22	0.984	No growth (NG)		0.19±0.14	17.12±5.81	0.92±0.70	8.52±1.75	NG	
	0.961	2.45±0.51	5.25±0.78	0.62±0.23	4.86±2.33	1.01±0.71	6.58±1.03	NG	
	0.936	2.58±0.89	5.53±1.10	2.74±0.22	6.68±0.60	0.92±0.27	6.34±4.41	NG	
	0.921	3.23±0.28	5.07±0.18	2.24±0.12	6.36±1.52	0.65±0.22	9.52±2.14	0.34±0.08	22.04±7.60
	0.892	3.15±0.15	4.74±0.18	2.31±0.14	5.48±0.46	1.63±0.16	2.21±0.33	1.29±0.30	18.90±9.20
	0.857	2.76±0.12	4.64±0.10	1.24±0.05	5.01±0.71	1.23±0.07	5.00±0.97	0.94±0.06	6.29±3.24
	0.826	1.51±0.08	3.43±0.34	0.70±0.08	6.69±1.96	0.56±0.02	8.83±5.52	0.48±0.14	14.18±2.35
30	0.984	5.37±0.51	1.77±0.72	2.62±0.21	1.65±0.30	1.24±0.82	5.06±0.17	NG	
	0.961	4.22±0.11	0.28±0.19	2.43±0.38	0.62±0.46	1.73±0.27	2.93±0.52	NG	
	0.936	6.92±0.06	1.55±0.11	4.39±0.61	1.11±0.14	3.52±1.15	1.16±0.66	0.28±0.10	5.03±4.02
	0.921	5.38±0.47	1.27±0.10	6.35±0.07	1.23±0.06	3.75±0.12	1.48±0.24	0.32±0.09	6.55±5.01
	0.892	6.09±0.36	1.88±0.07	4.12±0.11	0.97±0.12	4.24±0.08	1.51±0.15	0.13±0.06	7.79±4.41
	0.857	1.46±0.33	8.63±0.71	1.91±0.06	2.37±0.37	1.37±0.10	1.03±0.43	0.55±0.01	2.25±2.28
	0.826	0.17±0.23	1.65±1.27	0.44±0.05	1.35±0.94	0.54±0.04	2.81±2.09	0.49±0.03	2.93±0.64
37	0.984	4.87±0.42	1.17±0.08	5.71±0.93	1.26±0.09	0.72±0.23	0.82±1.13	NG	
	0.961	5.10±0.63	1.21±0.13	7.25±0.13	1.20±0.03	1.11±0.34	2.74±0.86	NG	
	0.936	5.66±0.25	1.19±0.06	6.62±0.04	1.19±0.04	2.64±1.01	1.18±0.65	0.19±0.18	9.54±11.80
	0.921	4.95±0.14	1.09±0.06	6.07±0.18	1.21±0.05	1.37±0.35	3.90±1.17	0.09±0.15	9.17±15.73
	0.892	3.30±0.53	2.25±0.16	4.03±0.20	1.06±0.08	2.48±0.05	0.83±0.49	0.29±0.15	24.37±11.79
	0.857	0.76±0.10	4.02±0.78	0.90±0.12	1.63±1.34	0.81±018	7.27±2.46	0.37±0.28	12.89±11.35
	0.826	NG		NG		0.40±0.63	3.84±0.00	NG	

8.3.2. Secondary modelling: individual effect of a_w or temperature on colony growth rate

The individual effect of a_w as a function of temperature on the colony growth rate of both fungal species was assessed using four secondary models. The parameter estimates of these developed models along with their standard errors, R^2 and RMSE are presented in Table 8-2. Estimated values of $a_{w,opt}$ using the Rosso square root (SQRT) cardinal model were 0.93 at 22°C and 30°C and 0.96 at 37°C for the *A. parasiticus* isolate. For UG AF06, $a_{w,opt}$ ranged from 0.91 to 0.95, while for UG AF35 it varied between 0.88-0.92 depending on the temperature. Lowest $a_{w,opt}$ was found for UG AF60 (0.66-0.87). The optimal a_w required for growth has shown to increase with increasing temperature in all the isolates, except for UG AF60. Evaluating both the Ratkowsky and Rosso cardinal models, the estimated $a_{w,min}$ for *A. parasiticus* varied from 0.41 to 0.62 at 22°C, 0.80 to 0.83 at 30°C and 0.74 to 0.84 at 37°C, while for *A. flavus* isolates it varied between 0.74-0.79 at 22°C, 0.79-0.83 at 30°C and 0.82-0.83 at 37°C. The estimated $a_{w,min}$ by Rosso SQRT model yielded an unrealistic value (0.41) for *A. parasiticus (*might be due to the model characteristics and type of growth data of this strain*)*. Most studies in literature reporting $a_{w,opt}$ and $a_{w,min}$ values used synthetic or simulation media to study and model the growth of *Aspergillus* spp. and therefore do not accurately represent the real capability of the strains to grow on a natural food substrate (Astoreca et al., 2012; Garcia et al., 2011). Mousa et al. (2011) estimated $a_{w,opt}$ and $a_{w,min}$ to be 0.99 and 0.83-0.85, respectively for *A. flavus* on paddy. Values of $a_{w,opt}$ at 0.97-0.99 and $a_{w,min}$ at 0.74-0.75 have been reported for *A. flavus* on pistachio nuts (Marín et al., 2012). The $a_{w,opt}$ values estimated in our study were rather lower than the values reported in these studies, however, these estimations probably depend on the type of strain, matrix as well as the type of models used.

Table 8-2. Estimated parameters (±standard error) for the secondary kinetic models used to describe the effect of a_w on the growth rate of *A. parasiticus* (UG AP542) and *A. flavus* (UG AF06, UG AF35 and UG AF60) isolates grown in whole black peppercorns.

Model	Parameters	UG AP542			UG AF06			UG AF35			UG AF60	
		22°C	30°C	37°C	22°C	30°C	37°C	22°C	30°C	37°C	22°C	30°C
Ratkowsky	$a_{w,min}$	0.55±0.1	0.81±0.01	0.78±0.01	0.78±0.01	0.81±0.00	0.83±0.00	0.75±0.61	0.81±0.01	0.82±0.00	0.74±0.14	0.67±0.2
	$a_{w,max}$	0.98±0.0	1.04±0.02	1.02±0.02	0.99±0.00	1.03±0.01	1.04±0.01	1.01±0.19	1.01±0.01	1.01±0.01	0.97±0.004	0.98±0.003
	b	4.92±1.2	111.10±243.7	16.56±2.4	23.16±9.6	5293±53.8*10E+4	43.32±6.17	56850±57.2*10E+5	108.08±264.5	20079±94.7*10E+5	11790.2±3.7*10E+7	1038.7±3.0*10E+6
	c	56.73±10.7	2.01±5.2	27.06±14.4	9.00±4.4	0.04±3.7	7.82±2.18	0.01±0.010	1.87±5.04	0.01±3.98	0.005±18.0	0.032±9.2
	R^2	0.914	0.843	0.911	0.865	0.868	0.991	0.589	0.747	0.852	0.708	0.902
	RMSE	0.183	0.294	0.190	0.299	0.218	0.187	0.129	0.268	0.418	0.288	0.094
Rosso cardinal	$a_{w,min}$	0.62±0.04	0.83±0.00	0.84±0.00	0.79±0.02	0.83±0.00	0.83±0.01	0.78±1018.36	0.82±0.01	0.83±0.00	0.77±1901.4	0.34±41804.0
	$a_{w,opt}$	0.92±0.01	0.93±0.00	0.95±0.00	0.91±0.01	0.92±0.00	0.95±0.00	0.88±0.02	0.91±0.01	0.91±0.00	0.87±0.02	0.66±3.4
	$a_{w,max}$	0.99±0.0	1.01±0.01	1.04±0.01	0.98±0.00	1.00±0.01	1.02±0.01	0.99±0.01	0.99±0.00	1.00±0.01	0.97±0.005	0.97±0.01
	μ_{opt}	3.22±0.1	6.21±0.3	5.53±0.10	2.38±0.10	4.96±0.22	7.14±0.12	1.07±0.09	3.77±0.18	1.97±0.12	0.81±0.11	0.81±3.5
	R^2	0.831	0.801	0.962	0.812	0.788	0.980	0.522	0.756	0.715	0.558	0.851
	RMSE	0.476	1.081	0.398	0.558	0.877	0.415	0.233	0.720	0.672	0.432	0.089
Rosso square root cardinal model	$a_{w,min}$	0.41±0.1	0.80±0.01	0.74±0.02	0.74±0.02	0.81±0.02	0.83±0.01	0.75±1015.96	0.79±0.02	0.82±0.55	0.74±1651.4	0.68±722.7
	$a_{w,opt}$	0.93±0.00	0.93±0.00	0.96±0.00	0.91±0.00	0.92±0.00	0.95±0.00	0.88±0.02	0.91±0.01	0.92±0.00	0.85±0.015	0.83±0.05
	$a_{w,max}$	0.98±0.00	1.03±0.01	1.03±0.02	0.99±0.00	1.03±0.01	1.06±0.01	1.01±0.01	1.01±0.01	1.01±0.01	0.97±0.004	0.97±0.00
	μ_{opt}	1.77±0.1	2.50±0.1	2.40±0.1	1.56±0.04	2.21±0.05	2.65±0.02	1.02±0.05	1.95±0.07	1.44±0.06	0.87±0.093	0.75±0.04
	R^2	0.921	0.848	0.921	0.872	0.869	0.992	0.589	0.753	0.852	0.708	0.902
	RMSE	0.175	0.289	0.179	0.298	0.218	0.178	0.126	0.265	0.429	0.291	0.094
Gibson	a_0	-2.38±0.31	-2.80±0.65	-0.02±0.36	-7.77±0.50	-2.95±0.55	-0.91±0.29	-3.75±0.81	-3.52±0.49	-3.63±0.64	-0.02±0.6	3.5±0.8
	a_1	24.07±2.4	41.03±5.0	19.24±2.8	57.31±3.9	37.97±4.3	29.04±2.5	20.84±6.2	36.95±3.8	30.66±5.5	0.43±4.9	-33.4±6.2
	a_2	-41.02±4.38	-87.32±9.07	-48.35±5.0	-94.96±7.0	-77.86±7.7	-69.51±5.0	-29.94±10.9	-70.90±6.8	-55.80±10.8	-3.6±8.8	57.3±11.1
	R^2	0.728	0.807	0.900	0.866	0.794	0.922	0.518	0.751	0.554	0.106	0.440
	RMSE	0.239	0.496	0.275	0.380	0.418	0.180	0.355	0.364	0.386	0.619	0.583

A $a_{w,min}$ of 0.83-0.85 was estimated by Rosso model for *A. flavus* growth in chilli powder extract agar (Marín et al., 2009), which shows much closer to our estimations at 30°C. Sautour et al. (2001a), reported $a_{w,opt}$ of 0.970 and 0.974 while Gibson (1994) reported 0.980-0.995 for *A. flavus* grown in an artificial growth medium. The different values reported in previous studies are probably due to the differences in substrate composition and the availability of nutrients that may affect the chances of fungal growth under marginal conditions (that might influence the minimum a_w for their proliferation) (Pardo et al., 2004), as the optimum growth rate is very much dependent on the substrate (Garcia et al., 2011). However, Marín et al., (1999) have reported that the nutrient composition has no effect on the fungal proliferation under optimal conditions. Graphical representation of the different fitted models to illustrate the individual effect of a_w on the growth rate at different temperatures are presented for an *A. parasiticus* (UG AP542) and *A. flavus* (UG AF06) isolate in Fig. 8-3 and 8-4, respectively.

Comparing the mathematical indices of all the models, the Rosso cardinal model using the square root of μ_{max} gave low RMSEs (0.094-0.429) and high R^2 (0.708-0.992) indicating that the model can best describe the individual effect of a_w on the colony growth rate of isolates of both *A. flavus* and *A. parasiticus* species at different temperatures; the experimental data points lie on or nearby the model surface (Table 8-2; Fig. 8-3 & 8-4). However, at 22°C due to higher variability (low R^2) in the growth of UG AF35 at some a_w values, a good fitting could not be obtained by any of the models applied. Next to the Rosso cardinal models, the Ratkowsky model could also satisfactorily describe the individual effect of a_w on the colony growth rate of both *A. parasiticus* and *A. flavus* isolates. The Gibson model failed to exhibit good fitting of the experimental results for some isolates and at some growth conditions. Other studies on modelling the μ_{max} of *A. flavus* strains have shown R^2 values above 0.97 using the Rosso μ_{max} cardinal model in different substrates (Marín et al., 2009; Astoreca et al., 2012; Mousa et al., 2011). Similar to our findings, Marín et al. (2012) also concluded from the RMSE values that the cardinal models were the most adequate to describe the individual effect of a_w or temperature on the radial growth of *A. flavus*. In a previous study on corn the RMSE value was estimated as 0.175 for the cardinal (square root of μ_{max}) model and it was indicated that the model can sufficiently describe the individual effect of temperature or a_w on the colony growth rate of the isolate (Garcia et al., 2011).

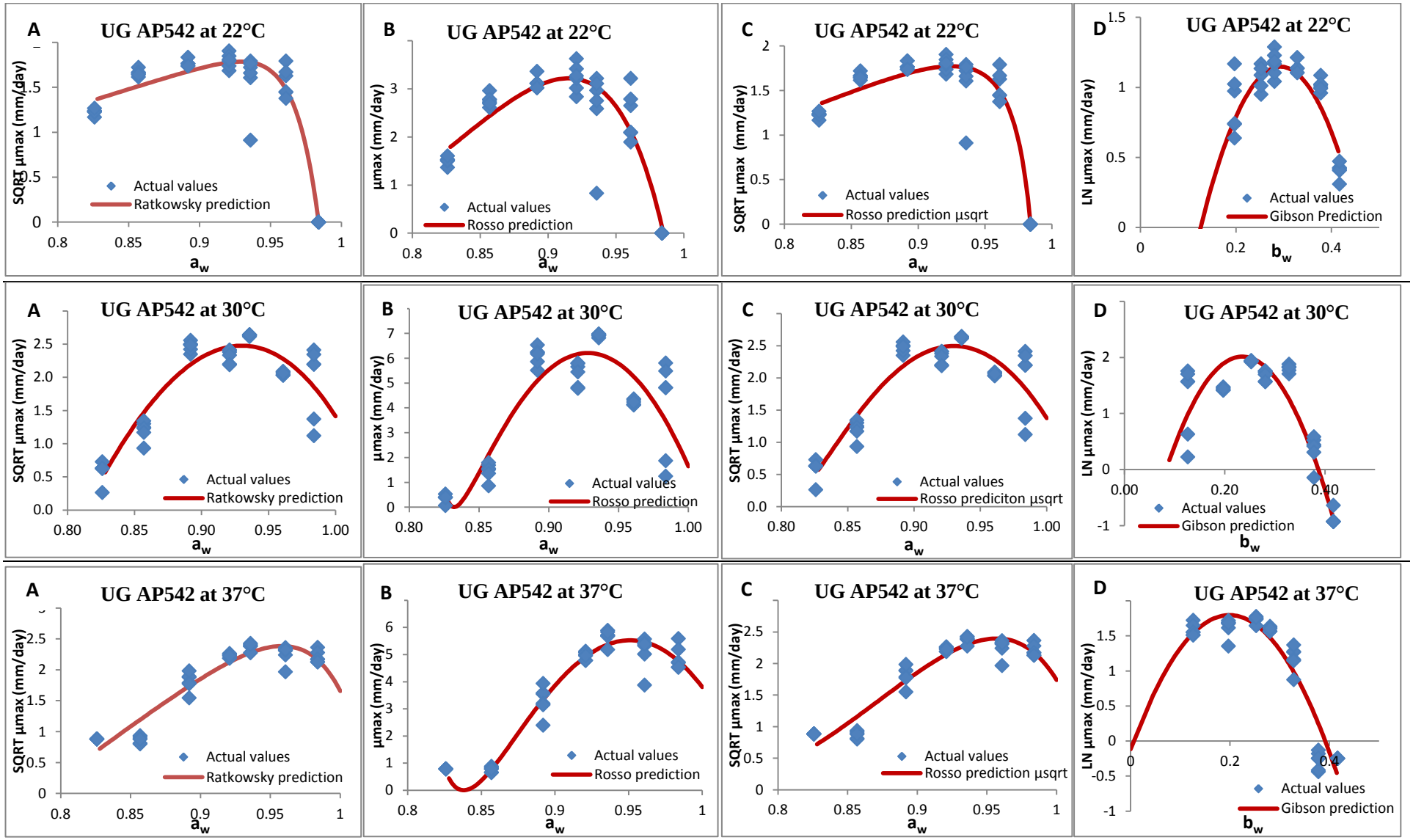

Fig. 8-3. Individual effect of water activity on the growth rate of *A. parasiticus* UG AP542 in whole black peppercorns at 22, 30 and 37°C; comparison of predicted (—) and actual (◆) radial growth rates between the secondary kinetic models [A] Ratkowsky, [B] Rosso cardinal, [C] Rosso square root cardinal and [D] Gibson.

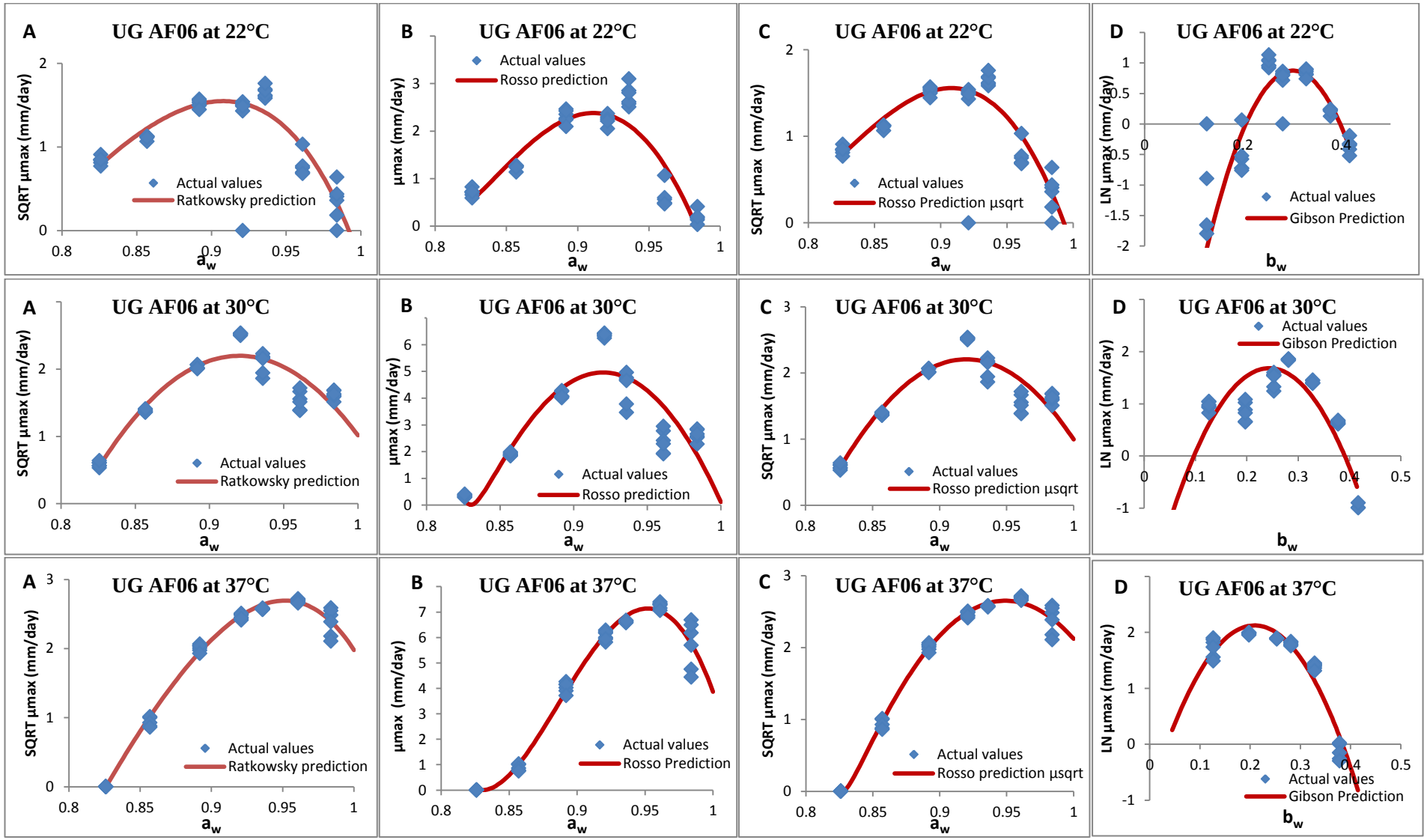

Fig. 8-4. Individual effect of water activity on the growth rate of *A. flavus* UG AF06 in whole black peppercorns at 22, 30 and 37°C; comparison of predicted (—) and actual (◆) radial growth rates between the secondary kinetic models [A] Ratkowsky, [B] Rosso cardinal, [C] Rosso square root cardinal and [D] Gibson.

8.3.3. Secondary modelling: combined effect of a_w and temperature on colony growth rate

Models with and without biological interpretations were used to explain the combined effect of a_w and temperature on fungal growth rates in black peppercorns. Coefficients of the models developed for each isolate are shown in Table 8-3. The minimal (T_{min}), optimal (T_{opt}), and maximal (T_{max}) temperatures as estimated by multi-factorial cardinal models for *A. parasiticus* were 12.5, 33 and 43°C, respectively. The values for the *A. flavus* isolates were 7-14, 29-33 and 39-54°C, respectively. These estimated T_{opt} values correspond with the optimum growth temperature values of 25-42°C reported by Klich et al. (2007) for *A. flavus*. T_{min}, T_{opt} and T_{max} of 13, 30 and 46-47°C, resepctively have been reported by Mousa et al. (2011) for *A. flavus* growth in paddy. Moreover, according to Pitt and Hocking (2009), *A. flavus* can grow over a wide temperature range of 10-48°C. In our case, rather low T_{min} (7°C) and high T_{max} (54°C) has been estimated for UG AF06 by this model.

Considering the a_w, $a_{w,min}$ of 0.80 was estimated for *A. parasiticus* by the multi-factorial cardinal model (Table 8-3), which is in the same range as the estimations by the Rosso model (0.74-0.84 at 30 and 37°C) that was used to explain the individual a_w effect. For the both *A. flavus* isolates $a_{w,min}$ estimations (0.81) were in between the range reported in Table 8-2 (0.68-0.83), which shows highly consistent estimations with Rosso squre root cardinal model. According to Pitt and Hocking (1999), $a_{w,min}$ for *A. flavus* growth are 0.78-0.81 or 0.84, while Marín et al. (2012) reported a $a_{w,min}$ of 0.74-0.75. Our model estimations agree with these reported $a_{w,min}$ values, despite the difference in substrate. On the other hand, $a_{w,opt}$ ranged from 0.93-0.96 for *A. parasiticus* and two *A. flavus* isolates. UG AF60 showed high growth variability at 37°C and no growth at many a_w conditions hence, modelling of the combined effect of a_w and temperature was not performed for this strain.

The estimated μ_{max} of *A. parasiticus* under the optimal a_w and temperature conditions for *A. parasiticus* was 1.92 mm/day while it ranged from 1.42-2.59 mm/day for the three *A. flavus* isolates as estimated by multi-factorial cardinal model (Table 8-3). A μ_{max} of 1.8 mm/day was reported in maize under optimal conditions (Garcia et al., 2011). Previous studies on *A. flavus* have estimated μ_{max} at 6.71-7.45 mm/day (Mousa et al., 2011) on rice and 14.54-14.72 mm/day (Marín et al., 2009) in chilli powder extract agar under optimal conditions. It is conceivable that these μ_{max} estimations are largely governed by the substrate, the growth conditions and the type of strain despite the type of model used for predictions. Anyhow, the major advantage of the multi-factorial cardinal model is that all the parameter estimates have a physiological meaning.

Table 8-3. Estimated parameters (± standard error), coefficient of determination (R^2) ('adjusted' R^2 within brackets) and root mean square error (RMSE) of the secondary models applied to explain the combined a_w and temperature effect on the growth response of *A. parasiticus* (UG AP542) and *A. flavus* (UG AF06 and UG AF35) isolates in whole black peppercorns.

Secondary models	Coefficients	Fungal strain		
		UG AP542	UG AF06	UG AF35
General polynomial	a0	-123.6±13.7	-169.1±14.1	-103.3±9.2
	a1	293.4±29.6	400.5±0.1	221.5±20.0
	a2	-174.1±16.2	-240.8±16.8	-125.5±11.0
	a3	-0.8±0.14	-0.96±0.1	0.26±0.1
	a4	-0.002±0.0	-0.004±0.0	-0.009±0.0
	a5	1.01±0.1	1.38±0.1	0.26±0.1
	R^2	0.730 (0.718)	0.822 (0.815)	0.707 (0.693)
	RMSE	0.399	0.397	0.496
Extended Gibson	a0	-13.3±0.8	-10.8±0.7	-8.9±1.0
	a1	49.9±1.9	46.3±1.7	21.4±2.5
	a2	-56.7±2.7	-54.0±2.4	-34.7±3.6
	a3	0.6±0.05	0.4±0.05	0.5±0.1
	a4	-0.01±0.0	-0.00±0.0	-0.01±0.0
	a5	-0.7±0.04	-0.7±0.04	-0.1±0.05
	R^2	0.894 (0.890)	0.916 (0.912)	0.625 (0.611)
	RMSE	0.247	0.219	0.507
Linear Arrhenius-Davey	a0	-153.4±15.1	-141.9±13.2	-111.4±9.4
	a1	327.6±33.2	312.5±29.0	229.7±20.8
	a2	-177.7±18.4	-169.0±16.0	-125.7±11.5
	a3	279.5±91.8	12.6±79.6	457.6±56.1
	a4	-4087.2±1249.5	-735.5±1083.8	-6297.2±764.8
	R^2	0.584 (0.570)	0.692 (0.682)	0.677 (0.665)
	RMSE	0.487	0.420	0.496
Multifactorial cardinal	T_{min}	12.5±5.1	6.7±1.7	13.8±2.3
	T_{opt}	33.1±1.0	32.9±2.9	28.5±3.0
	T_{max}	43.0±4.6	53.9±6.1	38.8±1.2
	$a_{w,min}$	0.80±0.01	0.81±0.01	0.81±0.01
	$a_{w,opt}$	0.93±0.01	0.93±0.01	0.96±0.01
	μ_{opt}	1.92±1.8	1.42±1.2	2.59±1.9
	R^2	0.709 (0.696)	0.800 (0.792)	0.762 (0.751)
	RMSE	0.949	0.972	0.665

The other models used in assessing the combined effect on growth were with parameters lacking any biological meaning. The 'adjusted' R^2 of all the models for *A. parasiticus* ranged from 0.696-0.890 (except for the linear Arrhenius-Davey) while for different *A. flavus* isolates it was 0.611-0.912. Performance of the different models in fitting to the experimental growth data is graphically shown for *A. flavus* and *A. parasiticus* isolates in Fig. 8-5 and 8-6, respectively. A line of equivalence (y=x) is drawn for better comparison of the deviations between the experimental and predicted μ_{max}. Moreover, the accuracies of the model predictions expressed as RMSEs (the lower the RMSE the higher the accuracy), followed the order, extended Gibson (0.219-0.507) > General polynomial (0.397-0.496) > Linear Arrhenius-Davey (0.420-0.496) > Multifactorial cardinal (0.665-0.972).

8.3.4. Model validation

The developed models were validated with independent experimental data obtained in black peppercorns. The performance of the models in terms of calculated bias (Bf), accuracy factors (Af), RMSE and SEP are shown in Table 8-4. Moreover, the performance of the validation data to different models developed for *A. flavus* and *A. parasiticus* are graphically shown in Fig. 8-5 and 8-6, respectively. Generally, Bf and Af assess the level of confidence in the predictions of the model and whether the model displays any bias that could lead to "fail dangerous" predictions. The Bf answers the question whether, on average, the observed values lie above or below the line of equivalence and quantifies the difference, the structural deviations (Te Giffel & Zwietering, 1999). Considering the *A. flavus* isolate, Bf ranged from 0.87 to 1.01 based on the predictions by different secondary models. A Bf<1 indicates that the mould grows slower than predicted by the model (predictions are faster), and is considered as "fail safe". All the examined models in this study had a Bf value equal or less than one. These Bf values are comparable to the previously reported Bf (1.0-1.1) for the models developed for *A. flavus* (Mousa et al., 2011 & 2013). A Bf ≤ 0.5 indicates a poor model, being too conservative in predicting growth rates that are twice as large as those of the experimental values. None of the validation models had a Bf<0.5 in our study, thus all the models are not overly conservative in predicting the *A. flavus* growth in black peppercorns. Moreover, a Bf≥1.1 indicates that the observed growth rate is larger than the predicted values at least by 10% (te Giffel & Zwietering, 1999). The extended Gibson model gave a Bf of 1.01, showing that the model gives the best prediction using the validation growth data; experimental growth rate will be only 1% larger than the predicted data.

Table 8-4. Mathematical indices (bias factor (Bf), accuracy factor (Af), root mean square error (RMSE) and standard error of prediction (SEP %)) used to evaluate the performance of the models describing the single and combined effects of a_w and temperature on the growth rates of *A. flavus* UG AF06 and *A. parasiticus* UG AP542 in whole black peppercorns.

Fungal growth models	UG AF06				UG AP542			
	Bf	Af	RMSE	SEP(%)	Bf	Af	RMSE	SEP(%)
Ratkowsky	0.980	1.014	0.020	4.10	0.872	1.147	0.063	17.78
Rosso square root cardinal	0.972	1.015	0.024	4.85	0.875	1.143	0.061	17.45
Linear Arrhenius-Davey	0.912	1.107	0.072	14.84	0.813	1.229	0.086	24.33
Extended Gibson	1.011	1.030	0.020	4.09	0.784	1.276	0.106	29.95
Second order polynomial	0.989	1.026	0.023	4.28	0.734	1.362	0.133	37.88
Multi-factorial cardinal	0.874	1.144	0.195	17.63	0.773	1.294	0.224	35.78

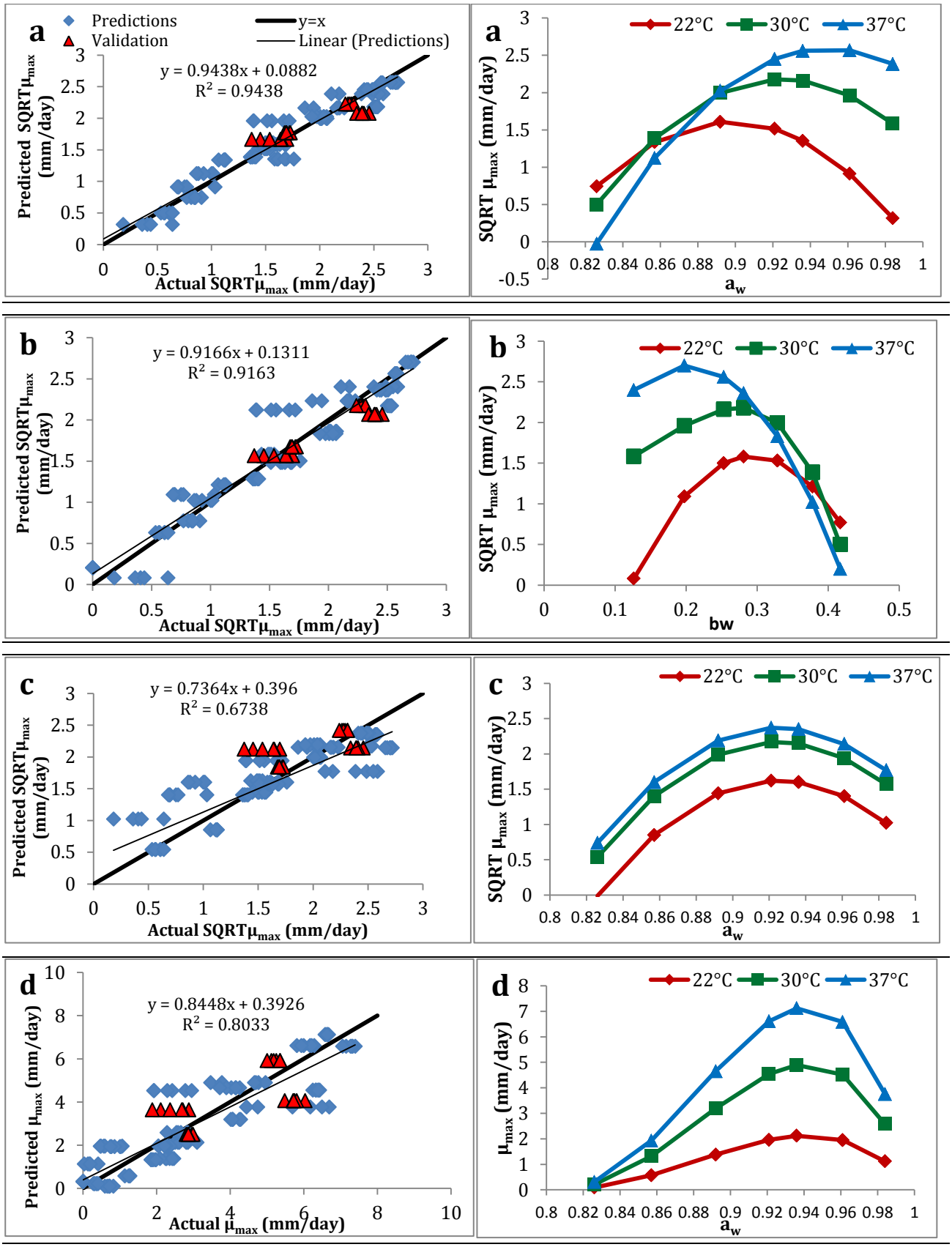

Fig. 8-5. Comparison of actual versus predicted μ_{max} of *A. flavus* (UG AF06) in whole black peppercorns using the combined a_w and temperature data of the models, a) polynomial; b) extended Gibson; c) Linear Arrhenius-Davey and d) multifactorial cardinal. Blue markers (◆) are the model development data and the validation data obtained at two a_w (0.88 and 0.91) and two temperature (30 and 37°C) conditions (6 points per condition) are shown as red markers (▲) in each models. On the right, change of μ_{max} predictions with a_w at different temperatures (22, 30 and 37°C) are shown for each developed models.

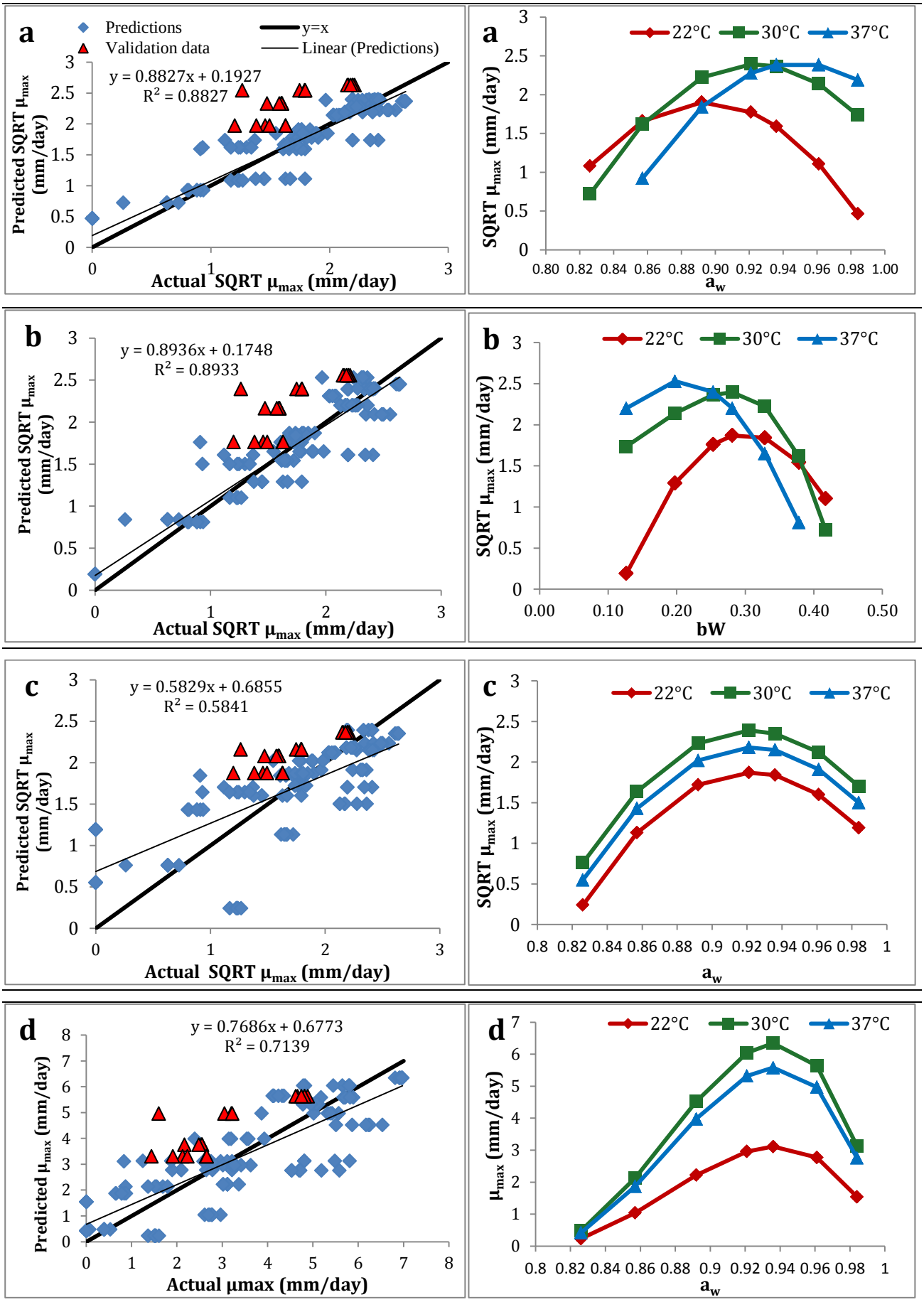

Fig. 8-6. Comparison of actual versus predicted μ_{max} of *A. parasiticus* (UG AP542) in whole black peppercorns using the combined a_w and temperature data of the models, a) polynomial; b) extended Gibson; c) Linear Arrhenius-Davey and d) multifactorial cardinal. Blue markers (◆) are the model development data and the validation data obtained at two a_w (0.88 and 0.91) and two temperature (30 and 37°C) conditions (6 points per condition) are shown as red markers (▲) in each models. On the right, change of μ_{max} predictions with a_w at different temperatures (22, 30 and 37°C) are shown for each developed models.

Since the Bf provides no indication on the average accuracy of the estimates (due to the fact that overestimation and underestimation tend to cancel out), the Af was also calculated. The Af averages the distance between each point and the line of equivalence as a measure of how close, predictions are to observations. The larger the Af value, the less accurate the average estimate is (Ross, 1996). The Af values for all the developed models for *A. flavus* UG AF06 ranged from 1.01-1.14, indicating that the average deviation between the predicted and observed values are small (1-14%), thus, guaranteeing better accuracies of the growth predictions by different models. The performance of these models are comparable with the Afs of the models previously validated for *A. flavus* growth in paddy (1.18-1.33) (Mousa et al., 2011), brown (1.124, 1.126) and polished rice (1.112, 1.113) (Mousa et al., 2013) and corn (1.05, 1.18) (Samapundo et al., 2007).

The RMSEs ranged from 0.019-0.195 and the SEP ranged from 4.1-17.6% for all the models for *A. flavus*. The multifactorial cardinal model showed comparatively high deviations between predicted and observed μ_{max} values (17.6%), which is also reflected in its rather high RMSE (0.195), however, it still had an adequate fit to the experimental data.

Considering *A. parasiticus*, the Bf of all the models ranged from 0.73-0.88, indicating that the models are conservative or "fail safe". Thus, *A. parasiticus* UG AP542 grows slower than the predicted growth rates, this can also be visualized in Fig. 8-6. Moreover, in this substrate *A. parasiticus* seems to grow slower than the *A. flavus* isolate used in the validation study as depicted by different models. The Af values indicate that the average deviation between the observed and predicted data ranged from 14-36%, higher than those obtained for *A. flavus*. The RMSE values were reasonably good for most of the models (0.061-0.133). Multi-factorial cardinal model showed rather high RMSE (0.224) which is also reflected in its rather high Af (1.29) and SEP (36%). A study on the growth of *A. flavus* in pistachio nuts using different kinetic models including cardinal models had RMSEs of 0.617-1.037 (Marín et al., 2012). Similar Afs (1.26-1.38) and SEPs (22.7-53.3%) values were reported by Baert et al. (2007), for *Penicillium expansum* growth in apples. In the same study predictably rather low bias and high accuracies (Af and Bf values closer to 1) were obtained in apple puree agar medium, since it is less complex compared to the real food matrices. Previously developed polynomial models had Afs of 1.12 and 1.13 for *A. parasiticus* and 1.128-1.380 for *Fusarium* spp., growth in corn (Samapundo et al., 2005b). Using independently derived data for validation, Tassou et al. (2007) reported rather high Bf (1.2-1.31) and Af (1.50-1.61) for all the models validated for *A. carbonarius* growth in synthetic grape juice medium. To our knowledge, only very few models have been developed for *A. flavus* and/or *A. parasiticus* on real food matrices and validated on the same matrix with independent data generated at different growth conditions (Samapundo et al., 2007c; Marín et al., 2012; Garcia et al., 2013). Developing models in culture media/simulation medium is considerably easier compared to doind the same on real food

matrices because of the complexity of the latter. Especially in our study, preparing a single compact peppercorn layer in Petri plates and maintaining its compactness throughout the study was not easy. Also attention has to be paid not to incline the plate during colony growth measurements, since slight disturbance on the pepper layer could influence the growth response of the fungal growth (disruption of the compactness of peppercorns will create gaps which will affect the fungal growth).

Comparing the performance of all the models on predicting growth of both fungal species, none of the models had a Bf≤0.5, thus none of them are overly conservative in predicting both *A. flavus* or *A. parasiticus* growth in black peppercorns under the limits of this study. The performance indices reveal that all the models are showing conservative predictions for the growth rate (0.5<Bf<1), with predicted μ_{max} greater than the observed μ_{max}. Moreover, as none of the models had a Bf>1.1, none of them were "fail dangerous". Hence, with certain degree of bias and accuracy, the developed models can be used to explain the growth responses of both of these mycotoxin producing *Aspergillus* species in black peppercorns. However, the Rosso square root cardinal model (Bf: 0.97, 0.88; Af: 1.02, 1.14) appears to be the best function to describe the individual a_w effect while the extended Gibson model (Bf: 1.01, 0.78 and Af: 1.03, 1.27) appears to be the best function for describing the combined effects of a_w and temperature on the growth rate of both fungal species in black peppercorns.

8.3.5. Mycotoxin production by *A. parasiticus* and *A. flavus* isolates in black peppercorns

Production of mycotoxins (AFG2, AFG1, AFB2, AFB1, STERIG and OMST) following the growth of *A. parasiticus* and *A. flavus* strains in black peppercorns at different temperatures and a_w conditions is shown in Tables 8-5 and 8-6, respectively. As mentioned earlier, all the studied isolates were proven to be toxigenic in malt extract agar but at different extents (up to ppm levels). Being secondary metabolites, the production of mycotoxins possibly follows a curve parallel to the growth with slight delay. However, regulation of secondary metabolism and the relationship between the rates of primary and secondary metabolism is still ambiguous (Calvo et al., 2002; Garcia et al., 2013). Adding to this complexity, very different results on mycotoxin production were revealed in this study. Considering *A. parasiticus* UG AP542, notably very small quantities of AFB1 (<LOQ-9.1 µg/kg) were produced at all a_w levels at 30°C. AFB2 was not produced in any of the growth conditions evaluated in this study. AFG2 and AFG1 were produced at very small quantities (<LOQ) only at 30°C, but not at all a_w conditions. However, STERIG was the predominant mycotoxin found, but OMST was not detectable at any of the growth conditions evaluated in this study. STERIG was detected in the a_w ranges of 0.826 to 0.961 at 22°C (20.3-76.7 µg/kg) and 0.892 to 0.984 at 30 and 37°C (<LOQ-63.5 µg/kg).

Table 8-5. Mycotoxin production (range, µg/kg) of *A. parasiticus* (UG AP542) after the growth in whole black peppercorns at different temperatures and a_w conditions (determined at the fungal colony diameter ~80-90 mm or when the growth stopped).

Mycotoxin	Temperature	a_w						
		0.984	**0.961**	**0.936**	**0.921**	**0.892**	**0.857**	**0.826**
AFG2	22°C				Not detected			
	30°C	ND[a] (0/3)[b]	<LOQ[c] (1/2)	ND (0/3)	ND (0/3)	ND (0/3)	ND (0/3)	ND (0/3)
	37°C				Not detected			
AFG1	22°C				Not detected			
	30°C	ND (0/3)	<LOQ (1/2)	<LOQ (2/3)	<LOQ (3/3)	ND (0/3)	<LOQ (1/3)	ND (0/3)
	37°C				Not detected			
AFB2	22/30/37°C				Not detected			
AFB1	22°C	NG[d]	<LOQ (1/3)	ND (0/3)	ND (0/3)	3.1 (1/3)	ND (0/3)	ND (0/3)
	30°C	<LOQ (1/3)	3.4, 9.0 (2/2)	3.3, 6.4 (2/3)	5.7, 8.1 (2/3)	3.4-3.7 (3/3)	3.1 (1/3)	ND (0/3)
	37°C				Not detected			
STERIG	22°C	NG	23.0-37.7 (3/3)	30.8-35.9 (3/3)	20.3-43.4 (3/3)	21.3-64.6 (3/3)	39.9-55.7 (3/3)	39.4-76.7 (3/3)
	30°C	22.7-27.9[e] (3/3)	27.7, 36.7 (2/2)	29.3-39.3 (3/3)	23.2-40.3 (3/3)	33.8-63.5 (3/3)	<LOQ (1/3)	ND (0/3)
	37°C	20.5, 39.4 (2/3)	23.6-36.4 (3/3)	26.7, 35.1 (2/3)	27.5-47.9 (3/3)	22.7-45.1 (3/3)	<LOQ (3/3)	NG
OMST	22/30/37°C				Not detected			

[a]Not detected; [b]Number of positives per total number of replicates; [c]Limit of quantification, LOQs of AFG2, AFG1, AFB1 and STERIG are 4.5, 5.0, 4.0 and 8 µg/kg, respectively; [d]No growth; [e]In case when only two samples were positive both values are given.

Considering the *A. flavus* isolates, UG AF06 was able to produce only STERIG (Table 8-6). It was produced at almost all the a_w conditions, 0.857 to 0.936 at 22°C (11-58.6 µg/kg) and from 0.857 to 0.984 at 30°C (12.6-40.6 µg/kg) and 37°C (<LOQ-43.1 µg/kg). Considering the effect of temperature on STERIG production by UG AF06, although the concentrations increased from 22 to 30°C with a slight decrease at 37°C, they did not differ significantly (p=0.747). However, STERIG was not produced in all the replicates even at the same growth condition (Table 8-6). Moreover, there seems to be no notable differences in STERIG production between different a_w values (especially at intermediate range 0.892-0.936) at a particular temperature or at different temperature at a particular a_w. These results are in agreement to the recent findings on the high frequency of STERIG in natural black pepper samples in this same research framework (Yogendrarajah et al.,

2014a). It is worth to mention here that, due to the structural similarities, aflatoxins and STERIG share prominent toxic effects, including genotoxicity and carcinogenicity (EFSA, 2013).

However, limited information on its occurrence and toxicity is available in contrast to aflatoxins. Moreover, similar to *A. parasiticus*, OMST was not detected on the peppercorns inoculated with *A. flavus* under any of the conditions evaluated in this study. Moreover, none of the mycotoxins were produced by the other two *A. flavus* isolates UG AF35 and UG AF60 at any growth conditions in peppercorns.

The absence or the production of mycotoxins at very small quantities in black pepper after heavy growth and sporulation of different toxigenic mould isolates was a notable observation. Generally, a strong relationship is found to exist between conidial development and secondary metabolite production. Poorly sporulating *Aspergillus* isolates were unable to produce aflatoxins (Klich, 2007; Kale et al., 1994). However, there seems to be no firm relationship between sporulation (based on visual observations not quantified) and mycotoxin production in black pepper.

Table 8-6. Mycotoxin production (µg/kg) of *A. flavus* isolate UG AF06 after their growth in whole black peppercorns at different temperature and a_w conditions (determined at the fungal colony diameter ~80-90 mm or when the growth stopped).

Mycotoxin	Temperature	a_w						
		0.984	**0.961**	**0.936**	**0.921**	**0.892**	**0.857**	**0.826**
AFB2	22/30/37°C	Not detected						
AFB1	22/30/37°C	Not detected						
STERIG	22°C	ND[a] (0/3)[b]	ND (0/3)	11-28.4 (3/3)	24.5, 32 (2/3)	39.4-42.4 (3/3)	21.6-58.6 (3/3)	ND (0/3)
	30°C	20.7, 27.7[c] (2/3)	12.6, 29.1 (2/3)	26.8-39.0 (3/3)	31.8, 39.8 (2/3)	26.8-40.6 (3/3)	22.2, 26.7 (2/3)	ND (0/3)
	37°C	7.6-23.4 (3/3)	25.4-42.1 (3/3)	31.7, 43.1 (2/3)	24.5-42.4 (3/3)	5.8-43.1 (3/3)	<LOQ[d] (3/3)	NG[e]
OMST	22/30/37°C	Not detected						

[a]Not detected; [b]Number of positives per total number of replicates; [c]In case when only two samples were positive both values are given; [d]Limit of quantification of STERIG is 8 µg/kg; [e]No growth.

In our study, even at optimum conditions aflatoxins were not produced by both fungal species in black peppercorns. Mousa et al. (2013), reported high levels of aflatoxins produced by *A. flavus* in polished and brown rice between 0.90 and 0.92 a_w at 20°C. In another study, optimal a_w for aflatoxins was reported to be 0.98 and optimal temperature was 25-30°C on paddy (Mousa et al., 2011). The optimal temperature for aflatoxin production by *A. flavus* and *A. parasiticus* can vary between 24 and 30°C (Klich et al., 2007). The differences in optimal a_w and temperature values for

aflatoxin production by *A. flavus* as predicted by other studies could be attributable to the differences in the type of substrate and fungal strains. Rather high levels of STERIG produced at lower levels of a_w or at lower temperatures suggest that the stress imposed at a temperature or a_w level that is lower than the optimal temperature or a_w for growth may initiate toxin production. Mousa et al. (2013) reported high levels of aflatoxins produced by *A. flavus* in polished and brown rice at a_w values between 0.90 and 0.92 at 20°C. The differences in optimal a_w and temperature values for aflatoxin production by *A. flavus* as predicted by other studies could be attributed to the differences in the type of substrate and fungal strains. However, there is lack of information on optimal conditions for STERIG production. Since it is the intermediate in the biosynthetic pathway of aflatoxins (Veršilovskis & De Saeger, 2010), optimal conditions for its production could be similar to that of aflatoxins.

Though, OMST production by the examined fungal strains was high in MEA (**Chapter 7**), it was not detected in black peppercorns under any of the growth conditions evaluated. Among the *A. flavus* strains, UG AF06 was able to produce STERIG, but not OMST or AFB1 in peppercorns. On the other hand UG AF35 and UG AF60 produced neither of these mycotoxins. Considering the fact that the transformation of STERIG to OMST is necessary for the production of AFB1, there might be an effect of black pepper components that probably deactivate/inhibit the enzymes needed for the bio-transformation of the precursor to AFB1. Generally, STERIG is rapidly transformed to OMST under optimal conditions. It could be that the pepper alkaloid piperine or other essential oils present in the peppercorns interfere at some points in the biosynthetic pathway of aflatoxins and block their transformation depending also on the strain sensitivity. These pepper components seem to have minimal effect on the growth of *A. parasiticus* and inhibitory effect on aflatoxin production (Madhyastha and Bhat, 1984). More research is needed to provide conclusive statements on this.

Though several studies reported production of aflatoxins by *A. flavus* over time in synthetic media or food, only very few studies tend to develop predictive models on its production (Mousa et al., 2011; Molina and Giannuzzi, 2002; Pitt, 1993). A model for OTA production by *A. carbonarius* in pistachio nuts has been proposed (Marín et al., 2008). Similar to other food substrates, production of mycotoxins in black pepper was also characterized by high inter- and intra-specific variability. Moreover, many other mycotoxins were not detected in this substrate even at highly favorable conditions. Hence, it was not possible to build a model which could predict the effect of the growth factors on mycotoxin production in black pepper at different a_w and temperature conditons. Moreover, each fungal strain exhibit a different inherent ability to biosynthesize mycotoxins (as it has been observed in STERIG production in this study) in a particular medium, thus the extrapolation of the models built with specific strains might not be representative for the species.

Mould growth in black pepper has shown relatively low intra-strain variability than mycotoxin production, thus the kinetics of the growth is better understandable than mycotoxin production. As highlighted previously, the best way to prevent spoilage and mycotoxin production might be through the prediction and prevention of fungal growth.

8.4. CONCLUSIONS

A kinetic relationship between the growth rate of the most significant producers of aflatoxins, *A. flavus* and *A. parasiticus* and environmental factors, temperature and water activity has been established in whole black peppercorns. The secondary kinetic models evaluated can be satisfactorily used with a good degree of precision and bias, to explain the individual and combined effect of a_w and temperature on growth rate of both of these fungal species in black peppercorns within the study limits. The examined secondary models could serve as reliable predictive tools for the prevention of fungal growth in black peppers. Inter- and intra-specific variability of mycotoxin production restricted the development of models for mycotoxin prediction. Absence or very low production of mycotoxins in peppers after heavy mould growth shows that there seems to be a significant role of pepper constituents in interrupting mycotoxin biosynthesis, with no or marginal influence on fungal growth and sporulation.

These findings provided stimulus to investigate the potential use of pepper extract to inhibit the mycotoxin production of the two potent aflatoxin producers. Thus, a preliminary study was performed to assess the effect of pepper extract concentrations on mycotoxin inhibition in order to establish a "minimum inhibitory concentration". The results are described in **Chapter 9**.

CHAPTER

9

ASSESSMENT OF BLACK PEPPER EXTRACT ON *Aspergillus flavus* AND *A. parasiticus* GROWTH AND MYCOTOXIN PRODUCTION: Towards a mitigation strategy

CHAPTER 9: ASSESSMENT OF BLACK PEPPER EXTRACT ON *Aspergillus flavus* AND *Aspergillus parasiticus* GROWTH AND MYCOTOXIN PRODUCTION: Towards a mitigation strategy

Summary

The effects of ethanolic and aqueous dilutions of black pepper extracts (11-2660 ppm) on the growth rate (mm/day), lag phase (days) and the mycotoxin production (aflatoxin B1 (AFB1), AFB2, AFG1, AFG2, sterigmatocystin (STERIG) and O-methyl sterigmatocystin (OMST)) of an *Aspergillus parasiticus* and four *Aspergillus flavus* isolates in malt extract agar were determined. The growth rate (mm/day) of all the fungal isolates increased linearly with decrease in pepper extract concentrations in both type of dilutions. There were no notable inter-species or intra-*A. flavus* species differences on the growth rate at a particular pepper extract concentration. The lag phase ranged from 0.18±0.49 to 2.27±0.15 days, depending on the fungal isolate and concentrations of extract. Production of mycotoxins was heavily influenced by the concentration of pepper extract. Very low STERIG (<LOQ) and rather high OMST production by all the isolates in both water and ethanolic dilutions show that there was no interruption in bio-transformation of STERIG to OMST, a crucial step in AFB1 biosynthesis in both fungal species. Moreover, most of the OMST produced was not bio-transformed to AFB1 at pepper extract concentrations above 83 ppm by *A. parasiticus* and at concentrations above 333 ppm by the two *A. flavus* isolates (UG AF06 and AF35). Notably, at high pepper extract concentrations despite growth occurring none of these metabolites were produced. A pepper extract concentration of 665 ppm could be suggested as the "minimum inhibitory concentration" for mycotoxin production (or <LOQ) for *A. parasiticus* and 333 ppm for *A. flavus*. However, the growth rate reduced only by 37% for *A. parasiticus* and 32-52% for the *A. flavus* isolates in both treatments at these concentrations and appeared to have only a minimal affect on sporulation. Prospective application of pepper extract as a mycotoxin mitigation strategy in agricultural commodities could be recommended and merits further research.

Keywords: *Pepper extract, Growth rate, Malt extract agar, Aspergillus flavus and parasiticus, Aflatoxins, O-methyl sterigmatocystin, Inhibitory concentration.*

Relevant publication: Yogendrarajah, P., Devlieghere, F., Jacxsens, L., De Saeger, S. and De Meulenaer, B. (2014). Assessment of black pepper extract on the growth and mycotoxin production of *Aspergillus flavus* and *Aspergillus parasiticus* isolates. In preparation.

9.1. INTRODUCTION

Moulds are ubiquitous microorganisms, able to colonize many kinds of food substrates and to proliferate under a wide range of environmental conditions. As mentioned earlier, approximately 5-10% of the agricultural products worldwide are spoiled by fungi, to the extent that they cannot be consumed by humans or animals. Besides, the FAO estimates that more than 25% of the agricultural produce is contaminated by mycotoxins (FAO, 2004). Hence, adequate control measures are necessary against mould growth and mycotoxin production to protect crops from spoilage and ensure that mycotoxin free food reaches the consumers.

Several strategies have been used over the years in controlling fungal growth and mycotoxin biosynthesis in stored agricultural commodities by chemical treatments with ammonia, acids and bases or by using a range of food additives and preservatives. The synthetic fungicides used in the field to control fungal diseases could leave residues that are harmful to health and environment. Restrictions imposed by the food industry and regulatory agencies on the use of some food additives and pesticides and the consumers growing demand for safe foods without preservatives have stimulated the interest in searching for natural antimicrobial compounds (Hammer et al., 1999; Chulze, 2010). Thousands of alternative indigenous products especially phytochemicals (essential oils or plant extracts) showing anti-microbial properties against phyto-pathogenic fungi have been reported by several researchers (Chulze, 2010; Nguefack et al., 2009; Bluma et al., 2008). The use of neem extract for inhibition of aflatoxin biosynthesis by *A. flavus* and *A. parasiticus* has been examined (Bhatnagar et al., 1990).

The previous chapter (**Chapter 8**) on the predictive modelling of fungal growth has shown growth and sporulation by *A. parasiticus* and *A. flavus* isolates in black pepper in most of the growth conditions investigated (a_w 0.826-0.984 and temperatures 22, 30 and 37°C). The growth rate and lag phase of the isolates differed depending on a_w and temperature values. However, mycotoxin analysis following the growth of these *Aspergillus* isolates in black pepper has revealed that there was no or significantly low concentrations of mycotoxins produced compared to its production in malt extract agar. This stimulated the interest to evaluate the potential of the pepper extract to inhibit mycotoxin production.

Therefore, the objective of this study was to assess the efficacy of pepper extract to inhibit growth and mycotoxin production of different *A. flavus* and *A. parasiticus* isolates in malt extract agar. In this way the toxigenicity of isolates in agar treated with pepper extract as well as the minimum concentration of pepper extract that could be used for the inhibition of mycotoxin production was determined. This might be a promising strategy in mitigating the mycotoxin problems in agricultural commodities.

9.2. MATERIALS AND METHODOLOGY

9.2.1. Chemicals, reagents and mycotoxin standards

Cyclohexane was obtained from Merck, Darmstadt, Germany. Ethanol was supplied by Chem-Lab NV, Zedelgem, Belgium. Other chemicals and reagents used were of analytical grade, same as described in **Chapter 2 (section 2.2.1).**

Mycotoxin reference standards (AFB1, AFB2, AFG1, AFG2, STERIG and OMST) used were the same as described in **Chapter 2 (section 2.2.2)** and **Chapter 7 (section 7.2.2).**

9.2.2. Growth medium, fungal isolates and fungal inoculum preparation

The basic growth medium used in this study was malt extract agar (MEA). Two mycotoxigenic fungal species, *A. flavus* and *A. parasiticus* were used. Both species were isolated from black peppers of Sri Lankan origin (more details in **Chapter 3 and 7**). One *A. parasiticus* (UG AP542) and four *A. flavus* (UG AF06, UG AF35, UG AF60 and UG AF62) strains were experimented. These isolates were found to be heavy mycotoxin producers in MEA as described in **Chapter 7**.

Fungal inoculum preparation was the same as described in **Chapter 7 (section 7.2.3).**

9.2.3. Extraction of pepper and inoculation

Ground pepper powder (100 g) was extracted twice with 200 mL of cyclohexane. The first extract was filtered after overnight stirring and a second extraction was performed for four hours. Both extractions were performed at room temperature. After filtering, the combined filtrate was evaporated using a rotavapor at 40°C (Annis et al., 2000). The extracted oily substance (2.66 g/100 g pepper) was diluted in 10 mL absolute ethanol (referred to as "raw" extract in the text). Exposure to light during the extraction was minimized as much as possible, to avoid the photo-isomerization of pepper alkaloids (e.g., piperine). A 200 µL aliquot of this raw pepper extract in ethanol was smeared on the top of the malt extract agar (20 g) and the plate (90 mm) was left open (~15min) until the ethanol had evaporated. After treatment, the plates were inoculated centrally with 10 µL of the spore solution (10^6 spores/mL) and incubated at 30°C. Agar treated with ethanol alone (200 µL) and untreated agar served as the controls. The experiment was performed in triplicate.

9.2.4. Preparation of ethanolic and aqueous dilutions of pepper extract

In addition to the "raw" pepper extract, inoculations were performed also with different dilutions of the pepper extract (in triplicates). For this purpose serial ½ dilutions of the "raw" pepper extract were prepared in ethanol to obtain the concentrations as shown in Table 9-1.

Table 9-1. Concentration of raw pepper extract and the different dilutions after treatment in malt extract agar.

Treatment	mg/plate	mg/kg (ppm)
"Raw" (D0)	53.2	2660
Dilution 1 (D1)	26.6	1330
Dilution 2 (D2)	13.3	665
Dilution 3 (D3)	6.65	333
Dilution 4 (D4)	3.33	166
Dilution 5 (D5)	1.66	83
Dilution 6 (D6)	0.83	42
Dilution 7 (D7)	0.42	21
Dilution 8 (D8)	0.21	11

In addition to the dilutions with ethanol, serial dilutions were also prepared in water (aqueous dilutions). The same experiment was performed with the ½ dilutions 1, 2, 3, 5 and 8 being prepared in water as opposed to ethanol. This experiment was performed to find out the possible influence of ethanol on growth and mycotoxin production by the different isolates evaluated in this study. The experiments were performed in duplicate.

9.2.5. Growth assessment

Fungal colony growth was measured as described in **Chapter 7 (section 7.2.4).**

9.2.6. Analysis of mycotoxins

The MEA was transferred to the extraction tube together with the fungal biomass, and extracted for mycotoxins (at fungal colony diameter ~80-90 mm) as described in **Chapter 7** (**section 7.2.5**).

The instrumentation (LC-MS/MS) and the conditions for aflatoxins and STERIG were the same as described in **Chapter 2 (section 2.2.5)** and for OMST the conditions were similar as described in **Chapter 7 (section 7.2.6).**

9.3. RESULTS AND DISCUSSION

9.3.1. Influence of pepper extract on the growth and lag phase of *Aspergillus* isolates

9.3.1.1. Growth and lag phase in ethanolic extract and ethanolic dilutions

Colony growth of the *Aspergillus* isolates in MEA treated with raw pepper extract and ethanol (control) are shown in Fig. 9-1.

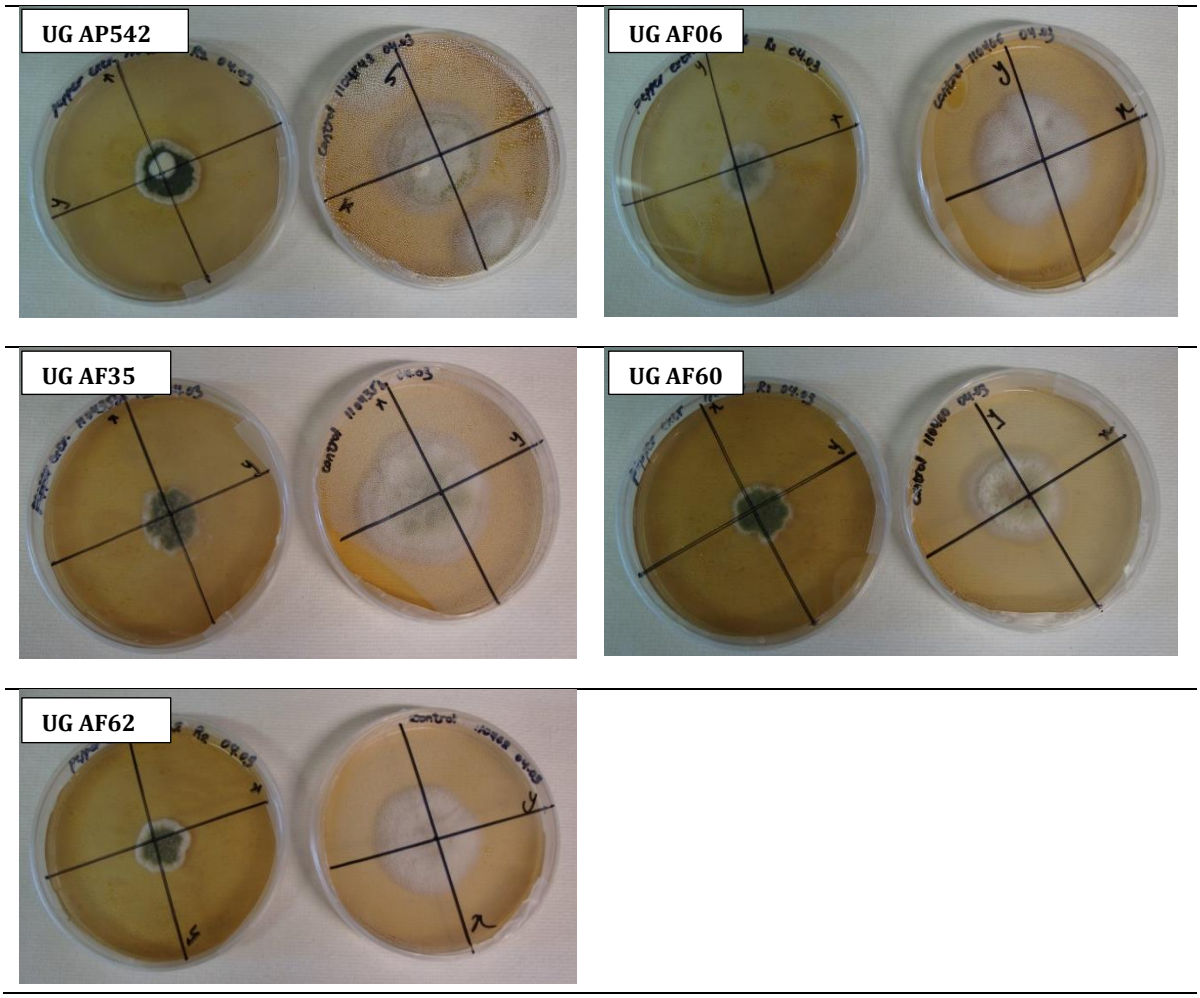

Fig. 9-1. *A. parasiticus* (UG AP542) and *A. flavus* (UG AF06, UG AF35, UG AF60 and UG AF62) growth in MEA treated with "raw" pepper extract showing slower growth and higher sporulation (left for each strain) compared with control (ethanol only) showing faster growth and less sporulation (right for each strain) after four days of incubation at 30°C.

Colony growth in pepper extract treated agar was slower than the control in all the isolates assayed. Generally, for a particular isolate the growth rate linearly increased with a decrease in extract concentrations (D1 to D8; 1330 to 11 ppm) regardless of the type of fungal species (Table 9-2A). Considering all the isolates the mean radial growth rate at 1330 ppm (D1) ranged from 2.63±0.05 to 3.25±0.61 mm/day while for the lowest concentration (11 ppm) it ranged from 5.5±0.18 to 6.1±0.09 mm/day. The growth rate at the lowest concentration (11 ppm) was almost comparable to that of the untreated (5.0±0.04 to 5.79±0.16 mm/day) or the ethanol treated control (5.46±0.46 to 5.87±0.17 mm/day) agar showing that the influence of pepper extract at this concentration on fungal growth

was almost insignificant. Moreover, there were no notable inter-species or intra-*A. flavus* species differences on the growth rate at a particular extract concentration. The mean lag phase ranged from 0.60±0.09 to 1.44±0.2 days for *A. parasiticus* and from 0.53±0.17 to 1.75±0.09 days for *A. flavus* depending on the extract concentrations and the isolate. Thus, fungal growth was initiated on the same day or the next day of incubation with all the dilutions for both species. Sporulation (not quantified) was observed with all the isolates, however it initiated at different days depending on the type of strain. Since, it was only a qualitative data further elaboration was not made.

9.3.1.2. Growth rate and lag phase in aqueous dilutions

The growth rates and lag phases of the different isolates in MEA treated with aqueous pepper extract dilutions were comparable to that of the ethanolic dilutions (Table 9-2). Similar to ethanolic treatments, generally the growth rate increased with an increase in extract dilution for a particular strain (except UG AF60 which showed higher variability among replicates). Considering all the isolates of both *Aspergillus* species (except UG AF60) mean radial growth rate at 1330 ppm ranged from 2.77±0.12 to 3.06±0.01 mm/day while for the 11 ppm it was 5.08±0.29-5.85±0.08 mm/day (Table 9-2B). The mean growth rate at the lowest 11 ppm was almost within the range to that of water treated (4.55-6.30 mm/day) agar (control) showing that the influence of pepper extract at this concentration on fungal growth was minimal. The mean lag phase ranged from 0.04±0.02 to 2.27±0.15 days, depending on the concentration of the extract and fungal species and the isolate. Initiation and extent of sporulation differed between different isolates, however, at the end of the experiment (after the colony reached the edge of the plate) all the isolates of both *A. parasiticus* and *A. flavus* were amply sporulated (only visualized not quantified) in all the dilutions. Overall, there were no significant differences in growth rate between both the types of dilutions (water or ethanol) for a particular fungal species or isolate (except UG AF60), suggesting that the influence of ethanol on the growth of these isolates was marginal compared to water ($p>0.05$; UG AP542 $p=0.539$, UG AF06 $p=0.675$, UG AF35 $p=0.462$ and UG AF62 $p=0.564$). However, it should also be mentioned that the dilutions with water were rather turbid compared to that of ethanol. This is probably due to the "precipitation" of piperine in water hence, the dilutions were more cloudy especially at the higher concentrations in water.

Table 9-2. Mean radial growth rate (μ_{max}, mm/day) and lag phase (days) (with standard deviations (SD)) of *Aspergillus parasiticus* (UG AP542) and *A. flavus* (UG AF06, UG AF35, UG AF60 and UG AF62) isolates grown in MEA at 30°C treated with different dilutions of pepper extract (concentrations in ppm are given in brackets) in A) ethanol (n=3) and B) water (n=2), compared with that of untreated (n=3). The dilutions 1, 2, 3, 5 and 8 are highlighted in treatment A for easy comparison with treatment B.

A. Treatment (ppm)	UG AP542				UG AF06				UG AF35				UG AF60				UG AF62			
	µmax (mm/day)		Lag phase (days)		µmax (mm/day)		Lag phase (days)		µmax (mm/day)		Lag phase (days)		µmax (mm/day)		Lag phase (days)		µmax (mm/day)		Lag phase (days)	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
D1 (1330)	2.93	0.11	0.60	0.09	3.25	0.61	0.53	0.17	2.87	0.14	0.55	0.09	2.66	0.01	1.31	0.01	2.63	0.05	0.85	0.06
D2 (665)	3.67	0.20	1.14	0.07	3.29	0.03	1.00	0.01	3.41	0.11	0.91	0.09	2.88	0.02	1.15	0.01	2.26	0.07	0.54	0.27
D3 (333)	4.53	0.24	1.44	0.20	3.98	0.03	1.32	0.02	3.89	0.08	1.03	0.05	3.33	0.22	1.46	0.17	2.64	0.91	0.84	0.41
D4 (166)	4.43	0.30	1.20	0.20	4.25	0.78	1.27	0.54	4.36	0.01	1.20	0.05	4.16	0.10	1.75	0.09	4.02	1.06	1.15	0.62
D5 (83)	5.92	0.12	1.33	0.18	5.33	0.11	1.43	0.02	5.44	0.15	1.39	0.14	3.85	1.22	1.02	0.12	4.11	0.53	0.95	0.41
D6 (42)	5.54	0.16	1.07	0.12	5.79	0.13	1.40	0.07	5.55	0.35	1.15	0.17	5.17	0.16	1.32	0.01	5.07	0.34	1.20	0.11
D7 (21)	5.50	0.89	1.40	0.10	6.04	0.21	1.22	0.07	5.10	1.14	1.03	0.14	5.46	0.11	1.18	0.05	5.30	0.22	1.01	0.00
D8 (11)	5.93	0.08	0.88	0.10	6.11	0.09	1.05	0.07	5.94	0.11	0.83	0.04	5.50	0.18	1.26	0.05	5.95	0.62	1.36	0.64
Control/ethanol	5.70	0.07	0.72	0.24	5.87	0.17	0.87	0.08	5.57	0.27	0.59	0.33	5.63	0.08	1.22	0.14	5.46	0.46	0.99	0.33
Untreated	5.21	0.16	0.85	0.05	5.79	0.16	0.90	0.05	5.49	0.38	0.86	0.07	5.00	0.04	0.85	0.08	5.30	0.64	0.80	0.14

B. Treatment (ppm)	UG AP542				UG AF06				UG AF35				UG AF60		UG AF62			
	µmax (mm/day)		Lag phase (days)		µmax (mm/day)		Lag phase (days)		µmax (mm/day)		Lag phase (days)		µmax (mm/day)		µmax (mm/day)		Lag phase (days)	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD[a]	Mean	SD	Mean	SD
D1 (1330)	2.77	0.12	0.55	0.34	3.06	0.01	2.02	0.20	2.88	0.32	0.77	0.54	1.71	1.66	2.91	0.32	2.27	0.15
D2 (665)	2.87	0.10	0.18	0.49	3.33	0.31	2.12	0.52	2.91	0.12	0.32	0.37	0.57	0.17	2.97	0.00	0.63	0.04
D3 (333)	4.07	0.18	0.28	0.20	3.64	0.34	1.90	0.85	3.23	0.02	0.04	0.02	1.92	1.90	3.81	0.08	0.88	0.06
D5 (83)	5.40	0.24	0.31	0.16	4.76	0.01	0.62	0.01	4.57	0.12	0.43	0.07	2.60	2.82	4.71	0.04	0.83	0.18
D8 (11)	5.53	0.05	0.26	0.11	5.56	0.28	0.50	0.18	5.08	0.29	0.52	0.10	0.51[b]	-	5.85	0.08	0.70	0.22
Control/H_2O	4.55	-	0.51	-	6.30	-	0.68	-	5.41	-	0.42	-	0.46	-	5.72	-	0.57	-

[a] Higher growth deviation was observed between replicates as could be seen with SD; [b] Growth observed only in one replicate.

9.3.2. Influence of pepper extract on mycotoxin production by *A. flavus and A. parasiticus* isolates

Production of aflatoxins (AFB1, AFB2, AFG1 and AFG2), STERIG and OMST by different *Aspergillus* isolates in MEA after treatment with raw, ethanolic and water dilutions of pepper extract was assessed when the colony reached a diameter of ~80-90 mm.

9.3.2.1. Mycotoxin production by *A. parasiticus* isolate

A. parasiticus UG AP542 produced none of the mycotoxins in MEA treated with "raw" pepper extract (Fig. 9-2). Production of secondary metabolites by this isolate under the different treatments is shown in Table 9-3. At the highest concentration (D1; 1330 ppm) AFG2, AFG1, AFB2 and STERIG were not produced in both aqueous and ethanolic treatments. In D1-water, production of AFB1 and OMST were <LOQ (2.6 µg/kg) but with D1-ethanol AFB1 was produced at levels <LOQ while OMST was produced at small concentrations (12-24 µg/kg). A similar observation was also found at 665 ppm of pepper extract for AFB1, but a rather high OMST concentration (675 µg/kg) was found in a replicate at the same concentration in ethanol. Generally, an increase in pepper extract concentration from 11 to 1330 ppm, decreased the production of most of the mycotoxins with complete inhibition being observed at high concentrations of both ethanolic and aqueous treatments. However, STERIG production remained at levels less than the LOQ (1.8 µg/kg) in all these treatments (11-333 ppm).

Compared to other toxins, AFG2 (<LOQ-26 µg/kg) and AFB2 (<LOQ-85 µg/kg) production was low in both type of dilutions. Production of these toxins was found to be rather low also in the control samples (only one replicate). Comparatively high concentration of AFB1 production started at 83 ppm (364 to 848 µg/kg) in both types of extract dilutions while in the controls it was 1137-2379 µg/kg. Moreover, the high concentration of OMST (left unconverted to AFB1) at 83 and 333 ppm of the pepper extract might be due to some interference caused by pepper components in the bio-transformation of OMST to AFB1. The control samples (and also at lowest pepper extract concentration of 11 ppm) in which rather low OMST and high AFB1 concentration were produced further support this observation. For comparison reasons, production of mycotoxins by the isolates in untreated MEA samples at substantially high concentrations is shown in Table 9-4.

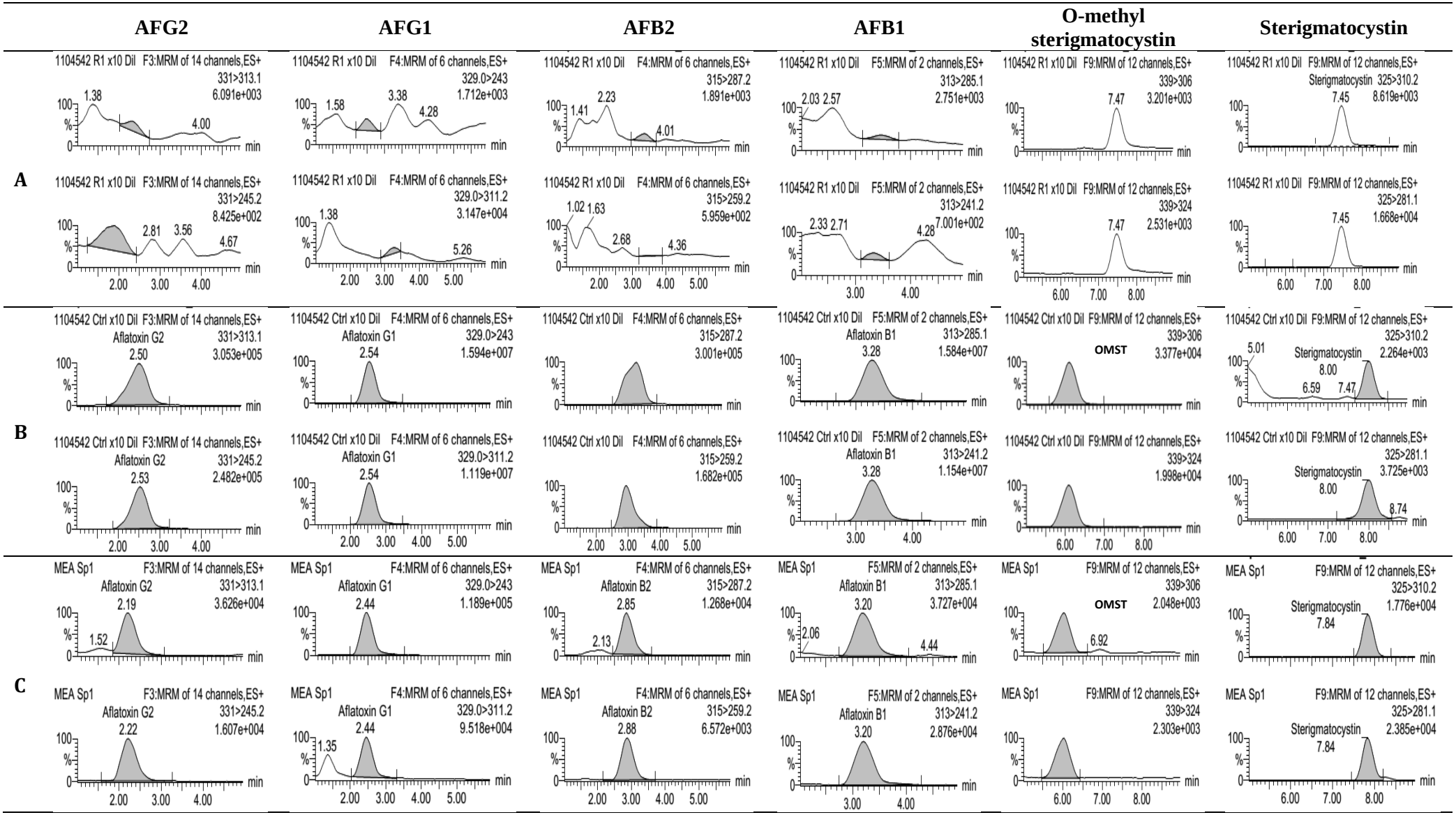

Fig. 9-2. MRM chromatograms of *A. parasiticus* isolate UG AP542 showing the absence of secondary metabolite production after inoculation in MEA treated with A) "raw" pepper extract and production of mycotoxins in B) MEA treated only with ethanol (control) and C) spiked agar.

Table 9-3. Mycotoxin production (µg/kg) by *A. parasiticus* isolate UG AP542 grown on MEA treated with "raw", water and ethanolic pepper extract dilutions at 30°C (colony diameter ~80-90 mm; results of both replicates R1 and R2 are given to better illustrate the variability in mycotoxin production).

Pepper extract concentration in ppm/replicates	Dilutions in water						Dilutions in ethanol					
	AFG2	AFG1	AFB2	AFB1	OMST	STERIG	AFG2	AFG1	AFB2	AFB1	OMST	STERIG
2660/R1, R2[a]	ND[b]	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
1330/R1	ND	ND	ND	<LOQ	<LOQ	ND	ND	ND	ND	<LOQ	12	ND
1330/R2	ND	ND	ND	<LOQ	<LOQ	ND	ND	ND	ND	<LOQ	24	ND
665/R1	ND	ND	ND	ND	<LOQ	ND	ND	ND	ND	<LOQ	31	ND
665/R2	ND	ND	ND	<LOQ	5.5	ND	ND	<LOQ	2.2	38	675	<LOQ
333/R1	<LOQ[c]	<LOQ	<LOQ	30	206	<LOQ	ND	4.9	4.9	83	281	<LOQ
333/R2	<LOQ	4.0	5.0	86	457	<LOQ	ND	<LOQ	<LOQ	12	115	<LOQ
83/R1	13	133	60	849	791	<LOQ	3.0	42	23	364	595	<LOQ
83/R2	13	133	56	784	744	<LOQ	6.5	82	44	673	986	<LOQ
11/R1	26	258	57	670	45	<LOQ	24	278	61	733	401	<LOQ
11/R2	12	120	20	239	7.1	<LOQ	25	274	85	1028	496	<LOQ
Control	55	501	103	1137	41	<LOQ	55	792	164	2379	13	<LOQ

[a] Replicates (R1, R2); [b] Non-detected; [c] Limit of quantifications for AFG2, AFG1, AFB2, AFB1, OMST and STERIG are 2.9, 3.6, 1.7, 2.6, 2.6 and 1.8 µg/kg, respectively.

Table 9-4. Mycotoxin production (µg/kg) by *A. parasiticus* and *A. flavus* isolates grown in pure MEA (untreated) at 30°C in all three replicates R1, R2 and R3 (colony diameter ~80-90 mm).

Strain ID	AFG2			AFG1			AFB2			AFB1			STERIG			OMST		
	R1	R2	R3	R1	R2	R3	R1	R2	R3	R1	R2	R3	R1	R2	R3	R1	R2	R3
UG AP542	458	301	146	2507	1947	1100	892	707	404	6005	5766	3539	43	73	60	821	1260	1208
UG AF06		NA[a]			NA		904	1146	138	7783	9667	6564	127	139	64	4380	7628	2018
UG AF35		NA			NA		768	702	404	6889	6121	6031	56	30	44	325	149	402
UG AF60		NA			NA		188	173	138	2573	2331	1960	29	21	14	504	337	256
UG AF62		NA			NA		96	19	7.9	1340	251	147	13	10	3.3	216	133	89

[a] NA, Not applicable; *A. flavus* isolates did not produce G aflatoxins.

9.3.2.2. Mycotoxin production by *A. flavus* isolates

Similar to the *A. parasiticus* isolate, none of the mycotoxins were produced by any *A. flavus* isolate in MEA treated with "raw" pepper extract. MRM chromatograms showing the absence of AFB1 production in pepper extract treated MEA by different *A. flavus* isolates compared with its presence in control samples are presented in Fig. 9-3.

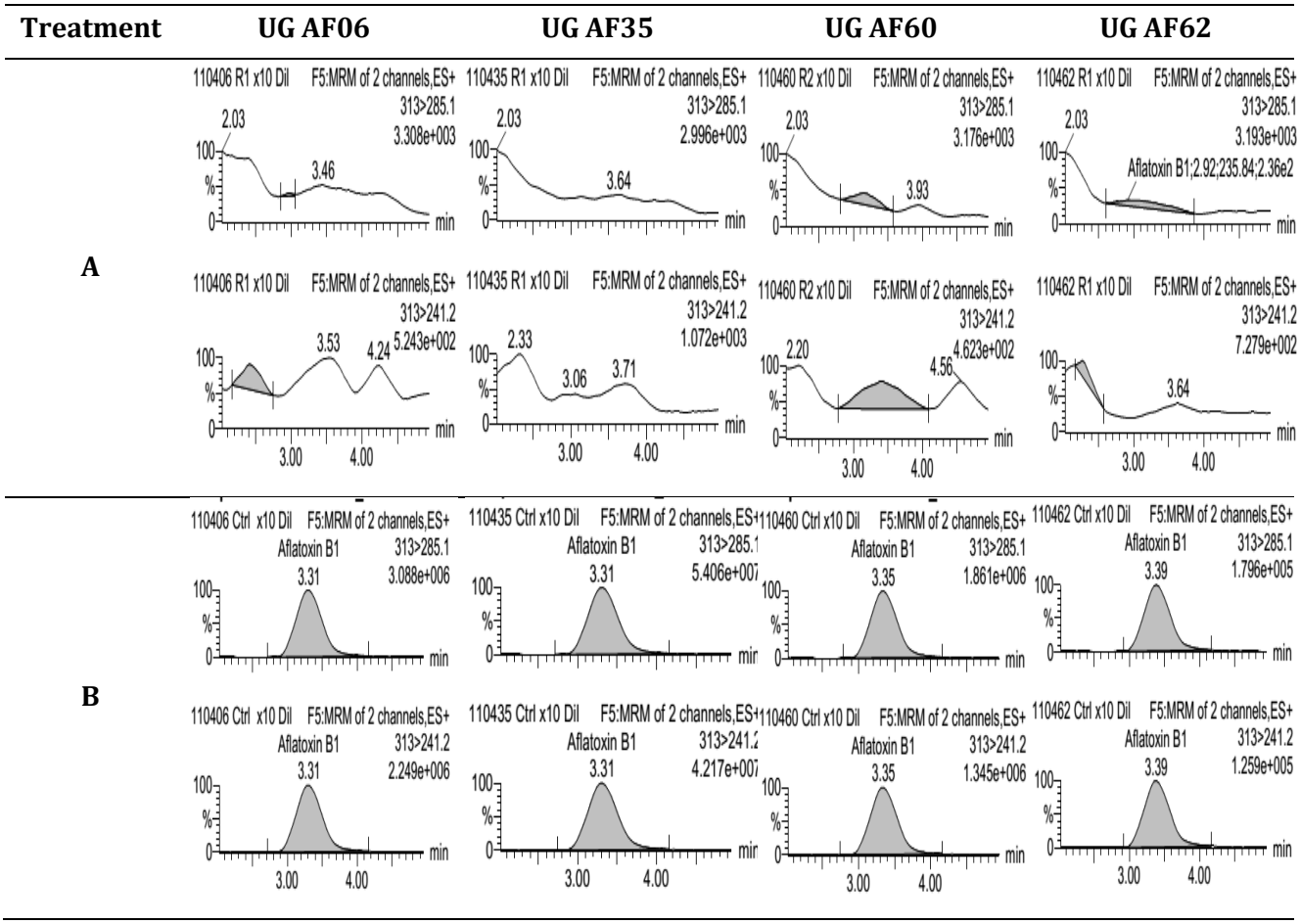

Fig. 9-3. MRM chromatograms of different *A. flavus* isolates showing A) no AFB1 production in MEA treated with "raw" pepper extract and B) AFB1 production in MEA treated only with ethanol (control).

The results on the production of mycotoxins by *A. flavus* isolates are given in Table 9-5. Considering the isolate UG AF06, none of the mycotoxins were produced in both water and ethanolic treatments until 333 ppm of pepper extract. At 11 ppm of pepper extract, a rather high OMST production was found with ethanolic treatments compared to water but STERIG concentrations were very low (<LOQ-3.9 µg/kg) in all the cases. The control experiment with water showed rather high AFB1 and OMST production compared to the control with ethanol. Similar to UG AF06, the isolate UG AF35 did not produce any mycotoxins (<LOQ to 3.6 µg/kg of AFB1 in few cases in water dilutions) in both type of treatments until 333 ppm pepper extract. At 83 ppm all the mycotoxins were produced, but STERIG concentration was very low as observed with the previous isolate.

Table 9-5. Mycotoxin production (µg/kg) by *A. flavus* isolates grown in MEA treated with "raw pepper extract (D0/2660 ppm), dilutions (D1/1330, D2/665, D3/333, D5/83 and D8/11 ppm) in water, ethanol and controls at 30°C (colony diameter ~80-90 mm).

A. flavus isolate	Concentrations in ppm/ replicates)	Water				Ethanol			
		AFB2	AFB1	OMST	STERIG	AFB2	AFB1	OMST	STERIG
UG AF06	2660/R1, R2[a]	ND[b]	ND	ND	ND	ND	ND	ND	ND
	1330/R1	ND	ND	ND	ND	ND	ND	ND	ND
	1330/R2	ND	ND	ND	ND	ND	ND	ND	ND
	665/R1	ND	ND	ND	ND	ND	ND	ND	ND
	665/R2	ND	ND	ND	ND	ND	ND	ND	ND
	333/R1	ND	<LOQ[c]	ND	ND	ND	ND	ND	ND
	333/R2	ND	ND	ND	ND	ND	ND	ND	ND
	83/R1	ND	4.4	46	<LOQ	<LOQ	36	478.2	7.9
	83/R2	<LOQ	22	135	<LOQ	<LOQ	16	268.5	<LOQ
	11/R1	39	597	1022	3.9	38	573	1494.7	<LOQ
	11/R2	20	187	61	<LOQ	34	475	1870.2	<LOQ
	Control	118	1597	2417	9.3	24	467	35.9	11
UG AF35	2660/R1, R2[a]	ND	ND	ND	ND	ND	ND	ND	ND
	1330/R1	ND	3.59	ND	ND	ND	ND	ND	ND
	1330/R2	ND	<LOQ	ND	ND	ND	ND	ND	ND
	665/R1	ND	<LOQ	ND	ND	ND	ND	ND	ND
	665/R2	ND	ND	ND	ND	ND	ND	ND	ND
	333/R1	ND	<LOQ	ND	ND	ND	ND	ND	ND
	333/R2	ND	ND	ND	ND	ND	ND	ND	ND
	83/R1	4.8	80	274	<LOQ	ND	<LOQ	15	<LOQ
	83/R2	46	696	323	5.7	<LOQ	37	90	<LOQ
	11/R1	<LOQ	6.1	85	<LOQ	22	356	278	4.2
	11/R2	42	659	355	<LOQ	39	619	290	4.4
	Control	110	1499	88	1.9	157	2474	283	3.6
UG AF62	2660/R1, R2[a]	ND	ND	ND	ND	ND	ND	ND	ND
	1330/R1	ND	ND	ND	ND	ND	ND	ND	ND
	1330/R2	ND	ND	ND	ND	ND	ND	ND	ND
	665/R1	ND	ND	ND	ND	ND	ND	ND	ND
	665/R2	ND	ND	ND	ND	ND	ND	<LOQ	ND
	333/R1	ND	7.9	ND	ND	ND	ND	ND	ND
	333/R2	ND	<LOQ	ND	ND	ND	ND	ND	ND
	83/R1	ND	26	<LOQ	ND	ND	<LOQ	ND	ND
	83/R2	ND	<LOQ	<LOQ	ND	ND	<LOQ	ND	ND
	11/R1	3.9	61	39	<LOQ	ND	ND	ND	ND
	11/R2	<LOQ	26	9.1	<LOQ	ND	<LOQ	<LOQ	ND
	Control	<LOQ	8.6	<LOQ	<LOQ	<LOQ	28	5.1	<LOQ

[a] Replicates (R1, R2) ; [b] Non-detected; [c] Limit of quantifications for AFB2, AFB1, OMST and STERIG are 1.7, 2.6, 2.6 and 1.8 µg/kg, respectively.

High concentration levels of mycotoxins were produced by this isolate in both control samples. In the control-water rather low OMST and high AFB1 production was found with this isolate similar

to the *A. parasiticus* isolate. Significantly low levels of mycotoxins were produced at all the pepper extract concentrations evaluated by isolate UG AF62, which appears to be a weak mycotoxin producer compared ot the other isolates. However, in contrast to the trends observed for the growth, higher variability in mycotoxin production was observed between replicates as highlighted also in chapters 7 and 8.

Minimum Inhibitory Concentration (MIC): Generally, mycotoxin production decreased with increasing concentrations of pepper extract for all the *Aspergillus* isolates studied in both type of dilutions. No notable difference in mycotoxin production could be observed between both water and ethanolic treatments at a particular concentration. Very low STERIG and rather high OMST production by all the isolates in water and ethanolic treatments showed that there appears to be no interruption in the bio-transformation of STERIG to OMST, which is necessary for AFB1 production. However, at the low pepper extract concentrations (11 and 83 ppm), not all the OMST produced could be converted to AFB1 as observed with the first two *A. flavus* isolates. This shows that there might be an optimum concentration of the pepper extract to interfere with this final step in AFB1 biosynthesis. Based on these initial experiments a concentration of 665 and 333 ppm could be suggested as a "minimum inhibitory concentration (MIC)" for inhibition of most mycotoxins (<LOQ) production by these aflatoxin producing *A. parasiticus* and *A. flavus* isolates, respectively. However, more replications and additional treatments are necessary to make strong conclusions.

General Discussion: Potential application of pepper extract for mycotoxin control in agricultural commodities needs further research. The influence of the pepper extract on sensorial properties of the commodity needs to be clarified and the economic feasibility of this substrate in mycotoxin mitigation should also be evaluated. However, compared with the controls the growth rate was only reduced by 37% for *A. parasiticus* and 32-52% for all the *A. flavus* isolates in both ethanolic and aqueous treatments at the MIC concentration. Thus, there is no complete inhibition of growth but rather slow compared with that of the untreated at the pepper extract concentrations applied. Concentrating the pepper extract even more (using mild dilutions instead of 10 times or by evaluating different extraction procedures) might help to completely inhibit the growth (preventing both mycotoxins the spoilage) since a decreasing trend in growth rate was observed with increasing the extract concentration. This needs to be studied.

According to the study of Madhyastha and Bhat (1984), piperine the major alkaloid of pepper has shown a 98% reduction in aflatoxin production by *A. parasiticus* only at 10,000 ppm. However, in the same study a rather low concentration of pepper oil (100 ppm) even stimulated the aflatoxin production. In our study the crude pepper extract used (which could contain several anti-oxidants/anti-microbial components at different proportions) even at such low concentrations (11-83 ppm), did not show a "stimulation" effect. Moreover, the aflatoxins produced in these treatments

were still much lower than those of the control samples in all the isolates. In the study of Annis et al. (2000), an alkaloid compound was isolated from black pepper (Cp2) which did not inhibit mycelial growth or β-tubulin gene expression but did inhibit the aflatoxin biosynthesis at the transcriptional level of an *A. parasiticus* isolate. However, the revealed inhibitory effect of pepper extract in our study may not be restricted only to this compound. It could be a combined effect of many other compounds with inhibitory effects, present in the pepper extract, which merits further research. Moreover, the extent of mycotoxin inhibition by a specified pepper extract concentration can also be strain specific, as it could be seen with the *A. flavus* strains investigated (Table 9-5). This could be a reason why there is still natural occurrence of aflatoxins found in peppers. Some strains could be highly resistant to pepper components thus they still be able to produce mycotoxins in the substrate. However, production of aflatoxins could not be restricted only to *A. flavus* and *A. parasiticus* but recent data indicate that aflatoxins could be produced by 13 species assigned to three sections of the genus *Aspergillus* (Varga et al., 2009). Possible application of the highly sensitive strains to pepper extract as a bio-control agent for mycotoxins inhibition could be studied as well.

Interestingly, there appears to be no correspondence between the observed spore formation and mycotoxin production following these pepper extract treatments. According to Klich, (2007) and Kale et al. (1994), generally a strong relationship was found between conidial development and secondary metabolite production, with poorly sporulating *Aspergillus* isolates not producing aflatoxins. However, in our study mycotoxins were not produced by both *Aspergillus* species even when there was heavy sporulation at high pepper extract concentrations. Additional investigations are necessary to elucidate the mechanism behind this interesting observation.

9.4. CONCLUSIONS

The growth rates of *A. parasiticus* and *A. flavus* isolates were influenced by the pepper extract concentrations in malt extract agar. In general, increasing the pepper extract concentrations decreased the growth rate as well as the mycotoxin production in both fungal species. It could be concluded that pepper extract at specified concentrations is a very efficient inhibitor of aflatoxin biosynthesis but surprisingly with only marginal effect on the growth and sporulation. Hence, actual mechanisms behind this inhibition need to be figured out. It might be that the components in the extract abruptly stop the biosynthesis in the early stages, they interrupt the enzymes involved in the synthesis or they disintegrate or react with the toxins to modify them into new compounds. Moreover, it could also be interesting to characterize the components present in the pepper extract, which could help to identify the major compound or many minor compounds responsible for the inhibitory effect. There seems to exist a great potential that the pepper extract could be used as a "natural solution" to mitigate the mycotoxin problem in agricultural commodities. Our findings could serve as early inputs to investigate further the usage of pepper extract in mycotoxins control in real food and feed applications.

CHAPTER 10

GENERAL CONCLUSIONS, RECOMMENDATIONS AND FUTURE PERSPECTIVES OF THE STUDY

CHAPTER 10: GENERAL CONCLUSIONS, RECOMMENDATIONS AND FUTURE PERSPECTIVES

This study is the very first to address the multi-mycotoxin issue in a food product in Sri Lanka. This research provides important insights in the mycotoxin contamination of spices and the associated risks in Sri Lanka, given the limited information available on the island. In this study, chilli and pepper (black and white) were studied since these two are the most important spices in world trade. From the overall findings of this PhD research, the general conclusions were made, future perspectives and emerging routes for further studies have been formulated and some suggestions for the government and stake holders are presented in this chapter.

Multiple mycotoxins in spices: *"It is true moulds and mycotoxins are everywhere, Sri Lankan spices are not exceptional"*

For a long time, mycotoxin analysis in spices was limited only to the "classical" mycotoxins, aflatoxins and ochratoxins. Recently, there were rising incidences of multiple mycotoxins in foodstuffs, hence there was a need to develop multi-analyte methods for different food matrices. However, analysis of mycotoxins is challenging since they often are present in low concentrations in complex food matrices like spices. Therefore in this research work firstly, a simple extraction method based on the QuEChERS approach was developed and successfully validated for the simultaneous determination of multiple mycotoxins using LC-MS/MS. The validated QuEChERS technique was applied on extremely complex and pigmented spices for quantitative screening of seventeen secondary metabolites, a major contribution in mycotoxin analysis in spices. As the name implies the method is quick, easy, cheap, effective, rugged and safe, therefore it is suitable for laboratories especially in developing countries seeking for a cost-effective sample preparation method. The method LOQ meets the maximum levels of the two regulated toxins aflatoxins (LOQ 2.3-4.7 µg/kg) and ochratoxin A (LOQ 4.2-13 µg/kg) in both spices hence, it can be used for the purpose of enforcement of the European Union maximum levels (5 µg/kg for AFB1, 10 µg/kg for total aflatoxins and 15 µg/kg for OTA (except 30 µg/kg OTA for *Capsicum* spp.) for all the spices). Moreover, the method is highly flexible for optimization and it is a widely used sample preparation technique for other food matrices, hence it can be further extended to include other modified secondary fungal metabolites and multi-residue analysis in spices.

Secondly, the developed multi-mycotoxin analytical method was applied on pepper samples collected from Sri Lanka. Apart from the aflatoxins (1-10%) and OTA (10%), STERIG (44%), FB1 (10%) and CIT (9%) were found to be present in peppers. In total, 63% of the black pepper samples were contaminated with at least one mycotoxin and 12% had more than two toxins. Moreover, the mycotoxigenic moulds were characterized both on their amount and frequency on the peppers. The present investigation revealed that there was a heavy contamination of toxigenic moulds on Sri

Lankan peppers despite the low water activity (a_w) and reported anti-microbial property of peppers. A total of 73% of the black pepper and 64% of the white pepper samples were contaminated with *Aspergillus flavus* and/or *Aspergillus parasiticus*. Although, Sri Lankan black pepper is known to have a high content (7-15%) of the anti-microbial substance piperine, the substrate supported well the growth of different toxigenic moulds. It is hard to establish a correlation between the observed a_w of the samples and mould or mycotoxin contamination since the contamination might also have occurred pre-harvest or during drying and storage. The high overall fungal contamination, high incidence of potential producers of mycotoxins and the presence of different fungal species showed that the peppers are a source of secondary fungal metabolites and might pose considerable public health risk.

Moreover, the application of the developed multi-mycotoxin method in chilli samples collected from Sri Lanka showed that chillies were frequently contaminated with several toxicologically significant mycotoxins. Compared to peppers the mycotoxin contamination in chilli was remarkably higher. The study revealed that 77% of the chilli samples were contaminated with AFB1 (<LOQ-687 µg/kg) of which 67% exceeded the EU ML of 5 µg/kg. OTA (<LOQ-282 µg/kg) was the second most prominent mycotoxin detected at 41% prevalence rate. Other mycotoxins detected included STERIG (38%), FB2 (15%) and CIT (8%). Co-occurrence of AFB1-OTA was found in 41% of the samples. Co-occurrence of mycotoxins was higher in processed chilli products than whole chilli pods. This high contamination of mycotoxins in chilli, emphasizes the necessity for routine analysis of this spice in Sri Lanka. Especially, in a developing country like Sri Lanka and other south and south east Asian countries where chilli is part of the daily diet, pre- and post-harvest measures should be implemented to keep these potent mycotoxins away from the table. This study needs authoritative attention from Sri Lankan legislative agencies as chronic exposure to even low mycotoxin levels could have long term detrimental health effects in humans taking into consideration its high usage in diverse Lankan cuisines. Acute intoxications due to AFB1 are also possible.

Risk assessment: *"Eating too much of chillies is not good for our health"*

The risk assessment study in Sri Lanka revealed that among the two spices, mycotoxin exposure associated with the consumption of chilli was higher than with peppers due to the high contamination and high consumption. Moreover, exposure was higher in the population from the North than in the South due to their higher chilli consumption. Margin of exposures associated with AFB1 were remarkably lower for chillies than for peppers indicating a significant public health concern for chillies in Sri Lanka. Thus, there could be a probable risk associated with developing aflatoxin induced liver cancer due to chilli consumption in Sri Lanka, depending on the prevalence

of hepatitis B virus infections. The risk characterization performed in this study for individual spices could be useful for the risk managers to directly set crop priority for adopting control measures. The data presented in this study indicated that there is a need for urgent concern to address the aflatoxin exposure due to chilli consumption. Furthermore, it is also important to evaluate the overall aflatoxin exposure through whole meal studies to estimate the overall incidence of AFB1 induced hepato cellular carcinoma (HCC) cases in Sri Lanka.

Chilli versus black pepper: *Why black pepper for predictive modelling?* This study discovered that there was a high mycotoxin contamination in chillies. Black peppers were found to be heavily contaminated with toxicologically relevant moulds while, the levels of mycotoxins found in the peppers were relatively low compared to chillies. Nevertheless, further work packages of this PhD research (Chapters 6-8) were dedicated to develop tools necessary for prediction of mould growth and their control in black peppercorns, mainly because of two reasons. Firstly, chilli produced locally in Sri Lanka is often consumed green. The very limited amount of dried chilli produced by the small scale farmers is used for their own consumption. The majority of the dried chilli consumed in Sri Lanka is mainly imported from India or China. Therefore, the mycotoxin problem found in dried chilli products could be considered as an import issue. However, the local processing and storage practices of the imported produce should never be neglected. In the absence of a systematic border checks and follow-up, it is often difficult to find where actual contamination started. Moreover, some predictive fungal growth models have already been developed for chilli powder to control the mould growth (Marín et al., 2009). On the other hand, there were no such studies present for black pepper. Secondly, the mould and mycotoxin problem addressed in black pepper in this study could be considered exclusively as a Sri Lankan issue. Black pepper is produced, processed and consumed locally. As mentioned earlier Sri Lanka is one of the major producers and exporters of black pepper hence, it is necessary that the peppercorns meet the regulatory measures (moulds and mycotoxins) of the importing countries, especially in the EU and USA. It has been suggested that the best way to prevent accumulation of mycotoxins is by preventing mould infestation. Therefore, the remaining work packages (Chapters 6-8) of this study focused on the development of tools necessary to ensure the safety and quality of Sri Lankan black peppercorns which could largely contribute to meet the regulatory standards to ensure it attracts premium value in the highly competitive global market and to protect the local consumers.

Ensuring safety and quality of peppercorns: *"Sri Lankan black gold should establish its supreme place in the world trade"*

Moisture sorption isotherms were developed in black peppercorns for both adsorption and desorption conditions. The sorption isotherms resulted in a shape of type III according to Brunauer-

Emmett-Teller (BET) classification. The EMC for desorption was generally higher than for the adsorption for a particular a_w indicating the occurrence of hysteresis. Amongst the eleven moisture sorption models evaluated, the Guggenheim-Anderson-de Boer (GAB) and Peleg model were found to be the best models to describe the sorption isotherms of black peppercorns over the temperature and a_w range studied. These unique isotherms developed in this study for black peppercorns could be useful in modelling of drying processes, design and optimization of drying equipment, predicting shelf-life stability, calculating moisture changes which may occur during storage and in selecting appropriate packaging material. High mould contamination in peppers was found even at low a_w (mean a_w 0.68±0.05 and 75% of the samples exceeded the European Spice Association a_w limit 0.65), however, the contamination might have happened during pre-harvest or during inappropriate drying and storage. Hence, drying of pepper should be further improved to bring down the a_w even lower and to be packed in appropriate packaging material. Controlling the a_w at one point in the supply chain cannot be a single solution to ensure the final product safety, but this needs to be followed up in all the steps along the farm-to-fork chain. A monolayer moisture content of 3.5-4.8% (dwb) could be specified as the optimal moisture content for the storage of whole black peppercorns at storage temperatures 22-37°C to ensure chemical as well as microbial safety. However, achieving this low moisture content is practically unlikely due to its strong binding to the matrix. Moreover, the sensorial properties (quality) of the peppercorns might be affected as well by lowering too much the moisture content. Hence, optimum a_w of ca. 0.60 (minimum a_w required for mould growth) can be targetted to ensure the safety of the peppercorns and this should be preserved until consumption. The black peppercorns can be stored at 22, 30 and 37°C, by reducing their moisture content to 10, 8 and 7%, respectively, with corresponding a_w values of 0.57, 0.59 and 0.56.

Toxigenicity study: *"Moulds: very beautiful but complicated creatures in the world to study"*

In the previous work package of this research, black peppers were found to be contaminated with several toxicologically relevant moulds. This prompted the interest to assess their mycotoxin producing potential at different temperatures. Therefore, a multi-mycotoxin method based on LC-MS/MS was developed and validated to assess the secondary metabolites production potential of fungal species grown on malt extract agar. The metabolite production was found to be very much temperature dependent, as well as strain specific for both *A. flavus* and *A. parasiticus* strains isolated from black peppers. High variability in mycotoxin production was found between replicates even under identical growth conditions. There were no correlations found between the growth rate and the production of secondary metabolites in both of these fungal species. However, notable correlations were observed between different secondary metabolites production in this growth substrate. A strong correlation between AFB2 and AFB1 was found despite the differences in their biosynthetic pathways in later stages. Low STERIG, high OMST and high AFB1 production

suggest that OMST could be used as a predictor for AFB1 production by both *Aspergillus* species. The developed method will be of great importance in the future for chemotaxonomic research and to study the conditions which could induce or suppress the complex secondary metabolism of various pure fungal isolates.

Predictive Mycology: *"There is always a solution to every problem"*

The imperious need for characterizing the effects of factors that govern fungal growth during pre- or post-harvest stages triggered the interest in developing mathematical approaches to describe and predict the fungal response to different growth factors in black peppers. In this work, a predictive kinetic relationship between the growth rate of the most significant producers of aflatoxins, *A. flavus* and *A. parasiticus* and environmental factors, temperature and a_w was established. The secondary kinetic models evaluated could be satisfactorily used with good degree of precision and bias, to explain the individual and combined effect of a_w and temperature on growth rate of both of these fungal species in black peppers within the study limits. The Rosso square root model can be recommended to describe the individual a_w effect while the extended Gibson model is the best model for describing the combined a_w and temperature effect on growth rate of these fungal species in black peppercorns. The examined secondary models can serve as reliable predictive tools for the prevention of *A. flavus* and *A. parasiticus* growth in black peppers. Inter- and intra-species variability of mycotoxin production restricted the development of models for mycotoxin prediction. The absence or very low production of mycotoxins in peppers in spite of heavy growth of mycotoxigenic isolates, showed that there might be a significant role of pepper constituents in interfering the mycotoxin biosynthesis at certain levels, with no or marginal influence on fungal growth and sporulation.

Mycotoxin mitigation strategy: *"Finally, a solution arose out of a problem"*

Finally, the role of pepper extract on *A. parasiticus* and *A. flavus* growth and mycotoxin inhibition was evaluated in malt extract agar. Production of mycotoxins was heavily influenced by the concentration of pepper extract. Pepper extract at specified concentrations is a very efficient inhibitor of aflatoxin biosynthesis but surprisingly with marginal effect on the growth and sporulation of both fungal species. This preliminary study showed that there is a great potential to use pepper extract as a mycotoxin mitigation strategy in agricultural commodities either during pre- or post-harvest. This could be possibly achieved by spraying or by placing slow releasing sachets with extracts in the storage bins. However, a systematic research is necessary on its efficient application and the economic feasibility need to be evaluated. This approach could be a practical and promising method for preserving stored products in villages and rural areas especially in developing countries where there is no access to modern storage facilitiies.

Recommendations: The government of Sri Lanka, relevant food safety authorities and stake holders in spice production and trade in Sri Lanka should consider the following measures to tackle the mycotoxin issue through the areas foreseeen (Fig. 10-1):

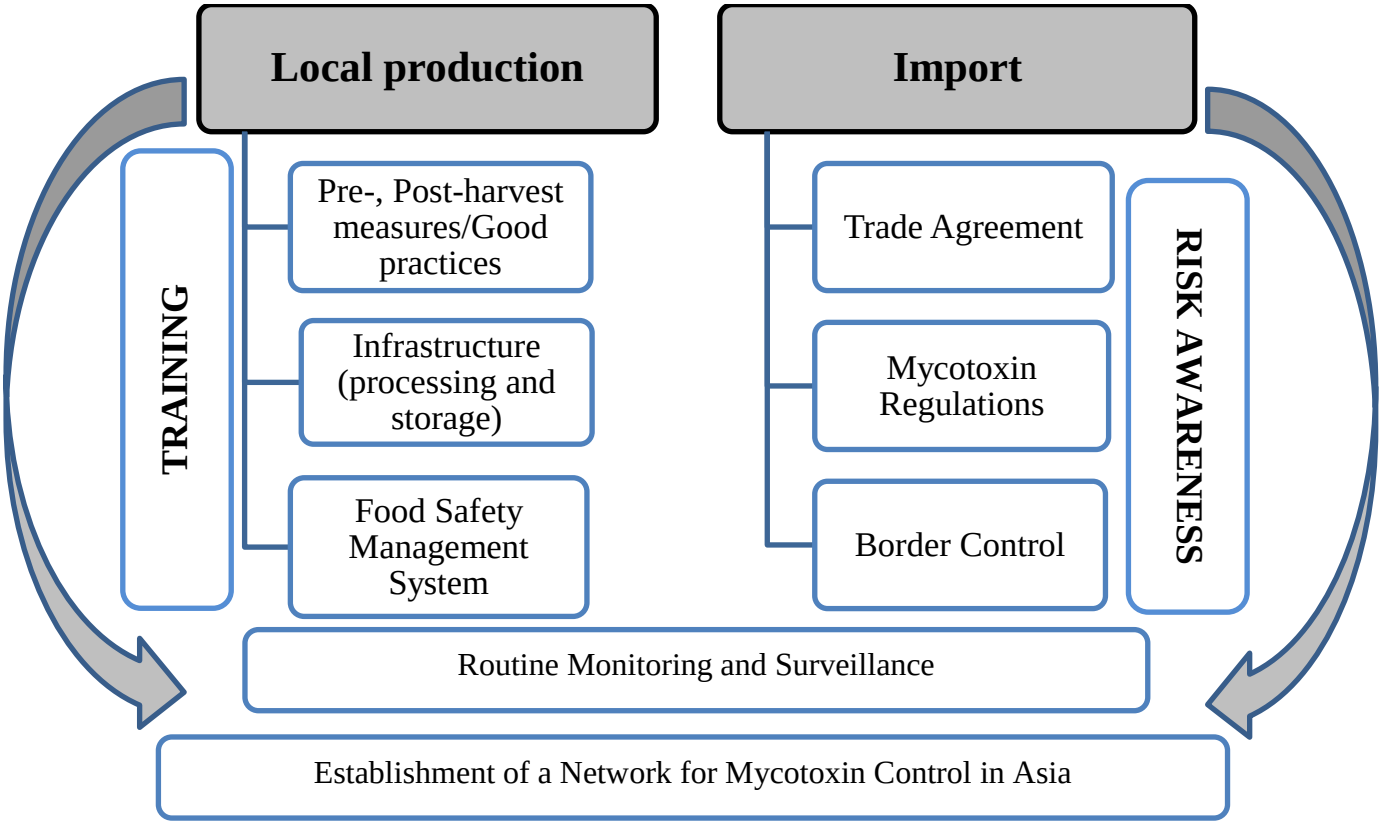

Fig. 10-1. Areas to emphasize in tackling the mycotoxin issue in Sri Lanka

- Improve pre- and post-harvest measures: Code of practice is intended to assist operators throughout the chain to apply good practices from farm-to-fork. The mycotoxin problem in spices seems to be generally a post-harvest issue (based on the existing studies on mould and mycotoxin contamination in spices.), however, possible contamination in the field cannot be ruled-out. Insect infestations, irrigation, fungicide application, inter-cropping, harvesting time, maturity at harvest etc., might influence mould contamination during pre-harvest. In Sri Lanka pepper vines are found to be affected by thrips and some sporadic diseases like, wilt, yellowing, leaf rot etc (**Chapter 1**). However, causal agents of these diseases generally do not affect directly the berries, rather they affect leaves, stem or root. Moreover, these moulds are not known to be mycotoxin producers. To our knowledge there are no comprehensive studies available on mould or mycotoxin contamination in spices that were from the field. Considering the spices, storage and processing are the major areas where contamination can be prevented while preventive measures in field need also be taken into account. Basic training to farmers and processors on drying could already be a driving step in reducing contaminations in both spices. Hence, better drying and/or storage facilities to farmers as well as to processors could be provided (by spice trade organizations) to store the dried spices in a controlled manner. A

food safety management system to ensure that the temperature and water activity of the storage facility is under control could be set-up, backed with inspections of local food quality control officers. Also revising the existing moisture limit of 12-14% (SLS) for peppers is recommended (according to the developed moisture sorption isotherm the corresponding a_w is 0.64-0.69 at 22°C; 0.72-0.76 at 30°C and 0.76-0.80 at 37°C), since they were heavily contaminated with diverse fungal species around this moisture limit (13.7±1.7%). However, it is difficult to predict when the actual contamination had occurred. Moreover, it is also important to consider achieving the required moisture content as fast as possible, thereby, liimiting the long term exposure to the environment. Considering the chilli, the importers and processors need to be advised to use good quality chilli pods for marketing, further processing (e.g., storage, sorting and packaging) and to discard the mouldy chillies. Morevoer, the traders need to be monitored not to sprinkle water to chilli pods. A simple case study would be very beneficial to illustrate how the results of this research work (predictive mycology models, moisture sorption isotherms etc.) could be taken on board in Sri Lanka and other countries in the same situation.

- Import agreements, mycotoxin regulations and border control: According to FAO, the importance of the development of internationally harmonized regulatory mycotoxin control measures that protect public health and promote fair trade at the international level cannot be overemphasized. However, it is the responsibility of the government to ensure that the commodity being imported is of good quality. In Sri Lanka currently a maximum level of 30 µg/kg is considered for aflatoxins for all the commodities (similar to India). Considering the fact that most of the dry chilli consumed in Sri Lanka is imported from neighboring countries and the very high mycotoxin contamination observed (67%>5 µg/kg of AFB1; 44%>10 µg/kg of total aflatoxins), the Sri Lankan government should start considering to enforce a more strict regulation for mycotoxins in chillies to ensure safe products entering the country. The results have revealed that even the existing regulations have not been strictly enforced in Sri Lanka (12% of the samples exceeded 30 µg/kg). The Sri Lankan government could consider borrowing EU regulations for aflatoxins as well as ochratoxin A for spices. However, even using the EFSA ML of 5 µg/kg for AFB1 and the lower bound deterministic scenario of chilli exposure, the MoE is still small, 1036 (according to EFSA, MoE<10000 is of public health concern). Therefore, the ML for AFB1 in Sri Lanka should be even lower than 1 µg/kg to minimize the exposure to this genotoxic carcinogen at the current chilli consumption level. Through the Ministry of Agriculture, Sri Lanka and the relevant government authorities (Department of Agriculture, Department of Export Agriculture - minor export crops) the

regulations could be stringently enforced to perform a systematic border control of the incoming consignments and to ensure compliance.

- Routine monitoring and surveillance: Monitoring programmes could play an important role in determining compliance and further having insights in the status of contamination. Therefore, the government could establish organized channels for routine monitoring and surveillance programmes through an efficient sampling plan (FAO or EU sampling guidelines). This could be best achieved through the establishment of properly equipped laboratories (important for regulation and to facilitate exports) with well-trained staff for both analytical and inspection activities, reliable analysis and sampling methods and application of analytical quality assurance programmes. Moreover, there is a need for a standardized method (especially for OTA and aflatoxins), so that the results could be accepted by buyers and sellers and proficiency testing should also be developed.

- Training programmes: Trainings could be organized by the Spice Council of Sri Lanka, Spice Export Development Board and/or Department of Agriculture to the food safety officials, farmers and processors on practical demonstrations on sampling, field detection by using rapid test kits for identification and analysis of various mycotoxins. A computerized data monitoring system should be developed which could assist in easy and fast risk communications hence, risk managers could take the necessary control or preventive measures on time.

- Mycotoxin awareness: It is also the responsibility of the government (and food safety authorities) to create awareness about the mycotoxin problem in Sri Lanka through proper risk communication. Farmers as well as the general public need to be informed about the possible health risks associated with mycotoxin exposure. Focus group discussions with farmers and processors could be arranged for lively and interactive discussions on mycotoxin issues. This will certainly help the households to improve the safety of their own food and ensure a healthy living. The government as well as non-governmental organizations (e.g., FAO) should help in raising awareness of health risks due to mycotoxins through leaflets, email alerts, mobile apps, posters or newspapers.

- A mycotoxin network in Asia: Regarding the mycotoxin issues there is a need for more coordination and streamlining of programmes in Asian countries. Since, India contributes 70% (FAO, 2011) of the global spice production it should consider intitiating such network. India is Sri Lanka's third largest trading partner globally, while Sri Lanka is India's second largest trading partner in the South Asian Association for Regional Cooperation (SAARC). Several countries in the continent have continuously reported mycotoxin occurrence in diverse food matrices over the years, however with limited studies on risk assessment. Most often mutual trade agreements have been signed among the Asian countries to facilitate food trade (exchange

of commodities e.g., Indo-Sri Lanka Free Trade Agreement (ISFTA, 1998)). Therefore, establishing an integrated network for mycotoxin regulation and control in Asia could be a promising strategy to tackle existing and emerging mycotoxin issues in the region. In this way the communication on the mycotoxin issues among the countries will become more effective similar to the RASFF system in the EU. The countries experiences and capabilities could be mutually shared to effectively tackle the mycotoxin issue, considering also the fact that the global warming being witnessed could have a big impact on mycotoxin occurrence in the future.

Finally, from a research scientist point of view, it could be interesting to extend this study in the following directions:

1) There is a need to explore the mycotoxin contamination in other Sri Lankan food products which could contribute higher mycotoxin exposures other than spices. Though parboiled rice is the staple food, bread and other wheat based foods (prone to mould infestation and mycotoxin contamination) are consumed in a substantial amount. A rather old study on parboiled rice (n=485) showed a very high contamination of AFB1 (60-186 µg/kg) and AFG1 (175-963 µg/kg) (FAO report, 1987), however, it has not been followed up. In addition to rice and wheat, nuts, coconut oil (copra used to extract oil, considered as a high risk commodity) and dairy products could be other possible sources of mycotoxin contamination to be explored. This could help in assessing the cumulative risk of a particular mycotoxin (or mycotoxins) due to the intake of combined food sources.

2) Risk assessment should further focus on combined mycotoxin exposure since combination of mycotoxins may lead to a higher and different toxicities than single mycotoxin exposure. Though, evaluating combined toxicity has been foreseen highly complex, novel strategies need to be identified to study the "end-toxicity" associated with these "cocktail effects" of multiple mycotoxin exposure. Furthermore, longitudinal cohort studies could be performed to incorporate aflatoxin biomarkers to measure aflatoxin exposure at individual levels. Aflatoxin metabolites (AFM1) in urine and aflatoxin-albumin adducts in blood can be used as biomarkers to assess the individual aflatoxin exposure which might show significant interaction with chronic HBV infection in HCC development. Hence, studies are needed on a large scale in different populations (especially in regions with high HBV prevalence) to provide a quantitative relationship between dietary aflatoxin intakes, biomarkers and HCC.

3) Further research is necessary to disclose the actual inhibition mechanism on aflatoxin bio-synthesis by pepper extract by using sophisticated instruments like high resolution mass spectrometry (HRMS). Possible hypotheses behind this inhibition mechanism could be directed towards following areas, fungal gene expression (changes in regulatory genes due to exposure

to pepper components), enzymatic inhibition (alterations in enzyme activity due to pepper extract) and detoxification of mycotoxins (modified metabolites or accumulation of other metabolites than that were studied). Hence, effective experimental set-up should be designed to test these hypotheses.

Furthermore, it could also be interesting to characterize the components present in the pepper extract (other alkaloids and amides than the major alkaloid piperine) showing inhibitory effect. These constituents could highly vary depending on the pepper cultivar, climatic conditions, maturity at harvest, processing method, storage conditions etc. Hence, a broad screening of different peppers originating from different pepper producing countries can be performed to characterize and quantify their bio-active/anti-microbial constituents showing strong inhibitory effects on mycotoxin production. Moreover, the role of pepper extract on growth and mycotoxin production of other toxicologically relevant fungal species, *Fusarium*, *Penicillium* and *Alternaria* spp. should be investigated in future.

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CURRICULUM VITAE

Pratheeba Yogendrarajah was born on 2nd of June, 1980 in Jaffna, Sri Lanka. In 2005, she obtained her Bachelor of Science (Hons) degree in Agriculture: Specialization Food Chemistry, with First class from Faculty of Agriculture, University of Jaffna, Sri Lanka. After graduation she started her career as demonstrator in food chemistry and later as assistant lecturer in biochemistry at the same university. In 2007, she has been granted with VLIR-UOS scholarship to pursue Master of Science in Food Technology at Ghent University and Katholieke University of Leuven (Inter-University Programme IUPFOOD) and graduated with great distinction in 2009. Her master thesis research was carried out at nutriFOODchem unit, Department of Food Safety and Food Quality, Ghent University and CART (Centre for Analytical Research and Technology), Mass Spectrometry Laboratory, Chemistry Department, University of Liège, Belgium. In 2010 she started working as Agricultural Development Specialist for a project for development planning at Japan International Cooperation Agency (JICA) in Sri Lanka. Since March, 2011 she has been working on her PhD project entitled "Farm to fork risk assessment of mycotoxins in spices, chilli and pepper produced and consumed in Sri Lanka" with financial support received from Special Research Fund (BOF) of Ghent University. The results of this research project have been published in a number of peer-reviewed scientific journals and were presented in several national and international symposia. During this period she also supervised two students for the fulfillment of their master thesis. She was also giving lectures for the International Training Programme (ITP) in Food Safety for the past three years.

PUBLICATIONS IN INTERNATIONAL PEER-REVIEWED SCIENTIFIC JOURNALS

PhD: Publications

1] Yogendrarajah, P., Jacxsens, L., Lachat, C., Walpita, C.N., Kolsteren, P., De Saeger, S. & De Meulenaer. B. (2014). Public health risk associated with the co-occurrence of mycotoxins in spices consumed in Sri Lanka, *Food and Chemical Toxicology*, 74, 240-248.

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PhD: Submitted/In preparation

5] Yogendrarajah, P., Samapundo, S., Devlieghere, F., De Saeger, S. & De Meulenaer, B. (2014). Moisture sorption isotherms and thermodynamic properties of whole black peppercorns (*Piper nigrum* L.). Submitted.

6] Yogendrarajah, P., Devlieghere, F., Njumbe Ediage, E., Jacxsens, L., De Meulenaer, B. & De Saeger, S. (2014). Toxigenic potentiality of *Aspergillus flavus* and *Aspergillus parasiticus* strains isolated from black pepper using an LC-MS/MS based multi-mycotoxin method. Submitted.

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8] Yogendrarajah, P., Devlieghere, F., Jacxsens, L., De Saeger, S. & De Meulenaer, B. (2015). Assessment of black pepper extract on *Aspergillus flavus* and *Aspergillus parasiticus* growth and mycotoxin production: Towards a mitigation strategy. In preparation.

Master Thesis:

Furan formation in baby food model sytems via vitamin C degradation and fat oxidation; Promoters: Prof. Bruno De Meulenar and Prof. Norbert De Kimpe.

MSc: Publications

Owczarek-Fendor, A., De Meulenaer, B., Scholl, G., Adams, A., Van Lancker, F., Yogendrarajah, P., De Kimpe, N., Eppe, G., De Pauw, E. & Scipoo, M.L. (2010). Furan formation from vitamin C in a starch based model system: influence of the reaction conditions, *Food Chemistry*, 121(4), 1163-1170.

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ORAL PRESENTATIONS

Food Integrity and Traceability conference (ASSET, 2014), Apr 8th-10th, 2014, Queens University, Belfast, UK.

Yogendrarajah, P., Van Poucke, C., De Meulenaer, B. & De Saeger, S., Method development and validation for multiple mycotoxins analysis in spices.

MYTOX-happening, Mar 11th, 2014, Faculty of Pharmaceutical Sciences, Ghent University, Belgium.

Yogendrarajah, P., Jacxsens L., De Saeger, S. & De Meulenaer, B., Co-occurrence of multiple mycotoxins in Dry Chilli (*Capsicum annum* L.) Samples from the Markets of Sri Lanka and Belgium and risk characterization.

Moulds Symposium, Mar 21st, 2014, Campus Schoonmeersen, Ghent university, Belgium.

Yogendrarajah, P., Deschuyffeleer, N., Jacxsens, L., Sneyers, P., Maene, P., De Saeger. S., Devlieghere, F. & De Meulenaer, B., Moulds in Spices-Special Focus in Black Pepper (*Piper nigrum* L.).

POSTER PRESENTATIONS

20th National Symposium for Applied Biological Sciences, (NSABS), Jan 30th, 2015, Louvain-la-Neuve, Belgium.

Yogendrarajah, P., Vermeulen, A., Jacxsens, L., Mavromichali, E., De Saeger, S., De Meulenaer, B. & Devlieghere, F., Mycotoxin production and predictive modelling kinetics on the growth of *Aspergillus flavus* and *Aspergillus parasiticus* isolates in whole black peppercorns (*Piper nigrum* L.).

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World Mycotoxin Forum (WMF), Nov 10th-12th, 2014, Vienna, Austria.

Yogendrarajah, P., Vermeulen, A., Jacxsens, L., Mavromichali, E., De Saeger, S., De Meulenaer, B. & Devlieghere, F., Predictive modelling kinetics on the growth and mycotoxin production of *Aspergillus flavus* and *Aspergillus parasiticus* isolates in whole black peppercorns (*Piper nigrum* L.).

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Yogendrarajah, P., Jacxsens L., De Saeger, S. & De Meulenaer, B., Co-occurrence of multiple mycotoxins in dry chilli (*Capsicum annum* L.) samples from the markets of Sri Lanka and Belgium.

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Yogendrarajah, P., Jacxsens L., De Saeger, S. & De Meulenaer, B., Co-occurrence of multiple mycotoxins in dry chilli (*Capsicum annum* L.) samples from the markets of Sri Lanka and Belgium.

35th Mycotoxin Workshop, May 22nd-24th, 2013, Campus Schoonmeersen, Ghent, Belgium.

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Yogendrarajah, P., Van Poucke, C., De Meulenaer, B. & De Saeger, S., Development of a QuEChERS based extraction method for the determination of multiple mycotoxins in spices using liquid chromatography coupled to triple quadrupole mass spectrometry.

Mycotoxin Analytical Symposium (MYCODAY), Mar 23rd, 2012, Liege, Belgium. Participation only.

GRANTS, SCHOLARSHIPS AND AWARDS:

2010: Special Research Fund (BOF) Doctoral Scholarship (2011-2015), Ghent University, Belgium.
2010: SEFOTECH.NUT, Erasmus Mundus programme recognized by European Commission.
2007: Masters Scholarship, Flemish interuniversity council (VLIR), University Development Cooperation Scholarship, Belgium.
2007: Masters Scholarship for Agro-ecology, University of Life Sciences (UMB), Norway.
2007: Masters Scholarship of Asian Institute of Technology, Thailand.
2005: R. R. Tharmaratnam memorial prize for the highest OGPA (3.82/4.00), Faculty of Agriculture, University of Jaffna.
2004: University award for the highest OGPA at the end of third year Bachelors programme, Faculty of Agriculture, University of Jaffna.
2002 & 2003: University prizes for the best performances in Agriculture, University of Jaffna.
2001 & 2002: University Scholarship for the best performances in first and second year in Agriculture, University of Jaffna.

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